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Molecular and Therapeutic Stratification of Endometrioid Ovarian Carcinoma

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By

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Declaration

I declare that this thesis is an original report of my research, has been written by me and has not been submitted for any previous degree. The experimental work is almost entirely my own work; the collaborative work have been indicated and acknowledged.

Due references have been provided on all supporting literatures and resources. I declare that this thesis was composed by myself, that the work contained herein is my own except where explicitly stated otherwise in the text, and that this work has not been submitted for any other degree or professional qualification.

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Abstract

Epithelial ovarian carcinomas (EOC) comprise five main histological subtypes, each displaying distinct pathological, molecular and clinical characteristics. Endometrioid ovarian carcinomas (EnOC) account for 10% of EOC and have been historically under-investigated. They typically present as early stage, grade 1 or 2 (low grade) tumours arising from endometriosis, and are associated with excellent clinical outcomes. However, Grade 3 (high grade) EnOC, as well as the even rarer de-differentiated carcinomas, can be challenging to differentiate from high grade serous ovarian carcinomas (HGSOC) based on morphology alone. Through the refinement of EnOC diagnostic criteria, several studies have now demonstrated that many previously diagnosed high grade EnOC are in fact HGSOC. This is further supported by gene expression profiling studies demonstrating that a proportion of high grade EnOC cluster together with HGSOC. As such, true high grade EnOC are increasingly rare and are associated with poor prognosis. WT1 immunohistochemistry (IHC) is a useful tool to discriminate high grade EnOC (WT1 negative ($WT1^{-ve}$)) from HGSOC (WT1 positive), reducing inter-observer variation.

To date, clinical and molecular characterisation of EnOC has been confounded by the inclusion of historically misclassified HGSOC in older studies. Mutational analysis performed by more recent studies have either only been applied to low grade EnOC, or lack information on grade or diagnostic criteria used. As a result, the molecular landscape and clinical behaviour of EnOC, in particular high grade EnOC, is not well defined.

In this study, tumours historically diagnosed as EnOC were identified through the Edinburgh Ovarian Cancer Database. Contemporary pathology review was performed utilising WT1 and p53 IHC. $WT1^{-ve}$ EnOC of all grades, and $WT1^{-ve}$ tumours with high grade serous and undifferentiated morphology were identified and included in the primary analysis. Clinical characteristics of the primary cohort were extracted from the database. Survival analysis was performed and responses to chemotherapy and endocrine therapy recorded. 63 tumours from the primary cohort underwent DNA extraction and whole exome sequencing (WES); comprising all $WT1^{-ve}$ tumours with mutant p53 expression on immunohistochemistry ($p53^{mut(IHC)}$) (n=28), all $WT1^{-ve}$ high grade carcinomas with p53 wild-type expression on immunohistochemistry ($p53^{wt\ (IHC)}$) (n=12) alongside a randomly selected subset of $WT1^{-ve}$ $p53^{wt\ (IHC)}$ low grade EnOC tumours (23 of 87 cases, 26.4%). Supervised mutational and copy number analysis was performed across 75 commonly mutated genes previously reported in endometrial, ovarian or pan cancer studies and molecular subgroups were identified. Unsupervised clustering analysis validated these molecular subgroups. Hormone receptor expression levels (oestrogen receptor (ER), progesterone receptor (PR) and androgen receptor (AR)) were evaluated as histoscores in the primary cohort. Multivariable survival

analysis, accounting for stage, residual disease, decade of diagnosis. grade and age, was performed on resulting molecular and hormone receptor subgroups.

Between May 1980 and December 2013, 125 $WT1^{-ve}$ tumours were identified. Overall five year disease specific survival (DSS) was 73.2% with the most favourable prognosis in those with early stage disease. Five year DSS in patients with advanced stage $p53^{wt\ (IHC)}$ low grade EnOC was 50.0%. Late relapses beyond five years were common in early stage disease. Patients with stage IV disease had poor prognosis with median DSS of less than one year. Radiological and CA125 response rates to platinum based chemotherapy in evaluable tumours was 44.5% and 69.3%, respectively. Median duration of endocrine therapy in evaluable tumours was 317 days (range 35 – 615 days). Of the 61 tumours successfully sequenced, *TP53* mutations *(TP53*^{mut}) were the most common (45.9%); followed by mutations in EnOC-associated genes (*ARID1A* (41.0%), *CTNNB1* (31.1%), *PTEN* (24.6%) and *PIK3CA* (23.0%)). Only *TP53*^{mut} status was independently associated with shortened DSS (HR=0.35, 95% CI 0.14-0.83, P=0.018). Copy number analysis revealed significantly more alterations in the *TP53*^{mut} tumours compared to *TP53*^{wt} tumours (P <0.0001), with a particular enrichment of variation across EnOC-associated genes in the *TP53*^{mut} subgroup with no EnOC-associated gene mutations. The majority of the primary cohort expressed ER and PR whereas AR expression was low. A PR histoscore of >150, when compared to a PR histoscore ≤ 150, was found to be independently associated with DSS, whereas no associations were observed with ER or AR expression levels. In particular, patients with stage II EnOC and a PR histoscore of >150 displayed a ten year DSS of over 90%.

Through this study, *TP53* mutation status and a PR histoscore of greater than 150 were identified as independent predictors of survival. This demonstrates EnOC to be a heterogeneous disease with distinct molecular and hormone receptor subgroups that demonstrate differential clinical outcome. Patients with *TP53* mutated and/or low PR expression EnOC have inferior prognosis and the development of novel therapeutic agents should be focused on these groups which display the greatest unmet need.

Lay Summary

Epithelial ovarian cancer was historically thought to be one disease and its treatment a 'one size fits all' approach comprised of aggressive surgery and platinum based chemotherapy. However, it is now recognised to be made up of five different diseases, namely, high grade serous (HGSOC) (80%), low grade serous (5%), endometrioid (EnOC) (10%), clear cell (10%) and mucinous ovarian cancers (3%). Each subtype has its own unique molecular origins, clinical behaviour and responses to treatment.

EnOC is a rare subtype of ovarian cancer which is usually diagnosed as early stage (disease confined to the ovary that has not spread), slow growing (grade 1 and 2) disease associated with excellent outcomes following surgery and chemotherapy. A small proportion are also diagnosed as fast growing (grade 3) disease which have a poorer prognosis. However, it can be challenging to tell the difference between the microscopic appearances of grade 3 EnOC and the most common subtype of ovarian cancer, HGSOC. Through refinements in the diagnosis of these tumours and the use of molecular testing, it is now recognised that many historically diagnosed grade 3 EnOC are in fact HGSOC. Studies have demonstrated that using Wilm's tumour 1 (WT1) antibody stains can help differentiate grade 3 EnOC (WT1 negative) from HGSOC (WT1 positive). The molecular profile, clinical outcomes and chemotherapy responses of EnOC, are therefore less well understood as many older studies were contaminated with misdiagnosed HGSOC. The aim of this study was to understand the clinical behaviour and genetic make-up of EnOC diagnosed using modern criteria, as well as to identify molecular prognostic factors which may help individualise treatments for patients.

In this study, historically diagnosed EnOC were identified between May 1980 and December 2013 through the Edinburgh Ovarian Cancer Database. Using modern diagnostic criteria, 125 EnOC were identified and clinical characteristics of these tumours were recorded. 63 tumours were sent for genetic profiling through a process called whole exome sequencing. Each tumour was stained for the presence of hormone receptors which include the oestrogen receptor (ER), progesterone receptor (PR) and the androgen receptor (AR).

Through this work, distinct molecular groups within EnOC were identified. Each of these groups displayed different clinical outcomes. Patients' whose tumours contained *TP53* mutations and low levels of PR staining did not survive as long as those without *TP53* mutations and high levels of PR staining. These two factors may thus provide additional prognostic information which may help to individualise treatments for patients. In particular, it may help identify a group of patients who may be able to avoid chemotherapy after surgery, sparing them the toxicities of treatment.

In conclusion, this study demonstrates EnOC to be a heterogeneous disease made up of different molecular and hormonal subgroups. Testing for *TP53* mutations and levels of PR may help guide the prognosis of patients with EnOC, however bigger studies are needed to confirm these findings. Further work should focus on the development of novel anti-cancer treatments for subgroups of EnOC which demonstrate the poorest prognosis.

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List of Abbreviations and Acronyms

AGO	Arbeitsgemeinschaft Gynäkologische Onkologie
AKT	Protein kinase B
APC	Adenomatous Polyposis Coli
AR	Androgen Receptor
ARID1A	AT-rich interacting domain-containing protein 1A
ATM	Ataxia-Telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related
BRAF	Proto-oncogene B-Raf and v-Raf murine sarcoma viral oncogene homolog B
BRCA	Breast cancer gene
BRG1	Brahma-related gene-1
CA125	Cancer Antigen 125
CA19.9	Cancer Antigen 19-9
CCNE1	Cyclin E1
CCOC	Clear Cell Ovarian Carcinoma
CDKN1A	Cyclin Dependent Kinase Inhibitor 1A
CDX2	Caudal Type Homeobox 2
CEA	Carcinoembryonic antigen
CK20	Cytokeratin 20
CK7	Cytokeratin 7
CN	Copy number
CR	Complete response
CSMD3	CUB And Sushi Multiple Domains 3
CTNNB1	Catenin Beta 1
DFS	Disease Free Survival
dMMR	Mismatch Repair Deficient
DNA	Deoxyribonucleic acid
DPC4/SMAD4	Deleted in Pancreatic Cancer-4/SMAD family member 4
DSS	Disease Specific Survival
EnOC	Endometrioid Ovarian Carcinoma
EnEC	Endometrioid Endometrial Carcinoma
EMSY	EMSY Transcriptional Repressor, BRCA2 Interacting
EOC	Epithelial Ovarian Cancer
ER	Oestrogen Receptor

ET	Endocrine Therapy
FAT3	FAT Atypical Cadherin 3
FBXW7	F-box and WD repeat domain containing 7
FFPE	Formalin fixed paraffin embedded
FIGO	International Federation of Gynecology and Obstetrics
GCIG	Gynecologic Cancer InterGroup
GOG	Gynecologic Oncology Group
H&E	Haemotoxylin and Eosin
HER2	Human epidermal growth factor receptor 2
HGSOC	High Grade Serous Ovarian Carcinoma
HR	Hazard Ratio
HRAS	Harvey rat sarcoma viral oncogene homolog
HRR	Homologous recombination repair
HRT	Hormone Replacement Therapy
IHC	Immunohistochemistry
IP	Intraperitoneal
IV	Intravenous
KRAS	Kirsten rat sarcoma viral oncogene
LGSOC	Low Grade Serous Ovarian Carcinoma
LS	Lynch Syndrome
MAPK	Mitogen-activated protein kinase
MuOC	Mucinous Ovarian Carcinoma
MEK	Mitogen-activated protein kinase
MLH1	MutL homolog 1
MLH3	MutL Homolog 3
MMR	Mismatch Repair
EORTC	European Organisation for Research and Treatment of Cancer
mRNA	messenger Ribonucleic Acid
MSH2	MutS protein homolog 2
MSH3	MutS Homolog 3
MSH6	MutS Homolog 6
MSI	Microsatellite instability
MSS	Microsatellite Stable
NACT	Neoadjuvant Chemotherapy
NF1	Neurofibromatosis type 1

NRAS	Neuroblastoma RAS viral oncogene homolog
OS	Overall Survival
PARP	Poly (adenosine diphosphate) ribose polymerase
PAX2	Paired box gene 2
PAX8	Paired box gene 8
PD	Progressive disease
PDL-1	Programmed cell death 1 ligand 1
PFI	Platinum Free Interval
PFS	Progression Free Survival
PI3k/AKT	Phosphatidylinositol 3-kinase/protein kinase B
PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
PIK3IP1	Phosphoinositide-3-Kinase Interacting Protein 1
pMMR	Mismatch Repair Proficient
PMS2	PMS1 Homolog 2
PPP2R1A	Protein phosphatase 2 scaffold subunits
PR	Progesterone Receptor
PR	Partial response
PTEN	Phosphatase and tensin homolog
RB1	Retinoblastoma 1
RECIST	Response Evaluation Criteria In Solid Tumours
RFS	Relapse Free Survival
ROCA	Risk of Ovarian Cancer Algorithm
RPL22	Ribosomal Protein L22
SD	Stable Disease
SEEC	Synchronous Endometrioid Endometrial Carcinomas
SEER	Surveillance, Epidemiology, and End Results
SET	Solid, pseudo-Endometrioid and/or Transitional-cell-like
SMAD3	SMAD family member 3
SMARCA4	SWI/SNF Related, Matrix Associated, Actin Dependent Regulator Of Chromatin, subfamily A, member 4
SMARCB1	SWI/SNF Related, Matrix Associated, Actin Dependent Regulator Of Chromatin, subfamily B, member 1
SWI/SNF	Switch/Sucrose Non-fermenting
TCGA	The Cancer Genome Atlas
TMA	Tissue Microarray

TMB	Tumour Mutation Burden
TP53	Tumour protein p53
UC	Undifferentiated Carcinomas
VEGF	Vascular endothelial growth factor
WHO	World Health Organisation
WT1	Wilm's Tumour 1

1. Introduction

1.1 Epithelial ovarian cancer

Epithelial ovarian cancer (EOC) is the most fatal gynaecological malignancy and the seventh most common cause of female cancers worldwide. In 2018, there were 295,414 newly diagnosed women worldwide with 184,799 deaths from this disease alone [1]. In the United Kingdom, EOC accounts for 4% of all new cancer diagnoses in females. As of 2016, 7500 new cases were diagnosed with a projected rise of 15% by the year 2035. The incidence of EOC increases with age with the vast majority of women diagnosed in their sixth and seventh decade in life.

Overall, survival in EOC has doubled over the last forty years with five year survival of 46% compared to 21% in the early 1970s (Figure 1). Stage is the most important determinant of survival with five year survival of stage I disease at 90% falling significantly to less than 10% in stage IV disease.

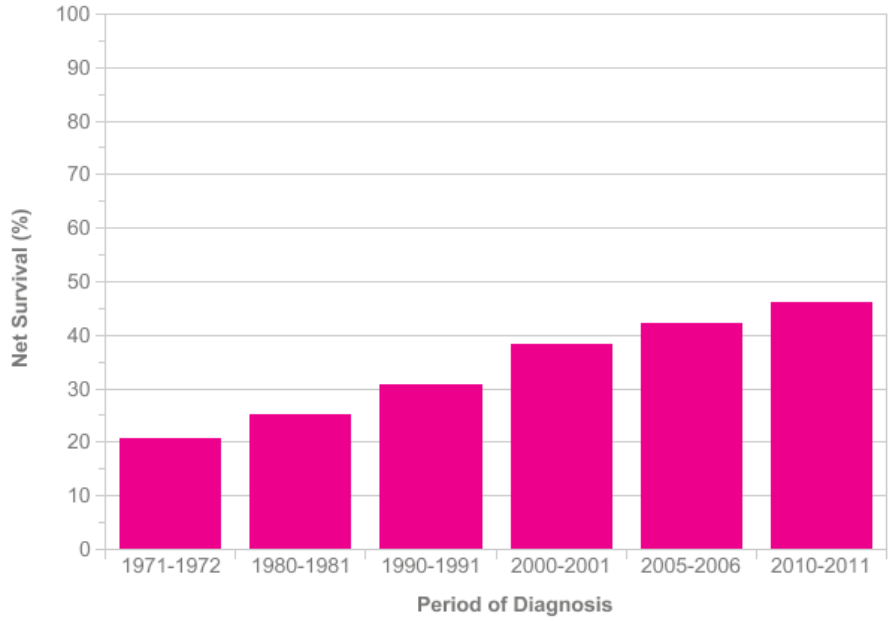

Figure 1: Five year age-standardised net survival for epithelial ovarian cancer (1971-2011)

(Cancer Research UK: URL: https://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/ovarian-cancer/survival#ref-2. Accessed January 2020.)

EOC is difficult to diagnose due to its location and non-specific presenting symptoms, as such most women with EOC will present at an advanced stage with poor prognosis [2]. The cancer antigen 125 (CA125) is a serum glycoprotein biomarker that is raised at diagnosis in 50% of women with early stage EOC and 85% of advanced stage EOC [2]. It can also be expressed by benign conditions such as ovarian cysts, pelvic inflammatory disease, endometriosis and uterine fibroids, and as such has a very poor positive predictive value of less than 5% when used as a screening test. Longitudinal measurements of CA125 have been shown to improve the detection of EOC compared with a defined threshold [3]. This led to the development of the Risk of Ovarian Cancer Algorithm (ROCA), incorporating serial CA125 levels, age and statistical risk of having a rapid rise in CA125 above baseline, to assist in tailoring the frequency and type of investigations [4]. The UK Collaborative Trial of Ovarian Cancer Screening compared multimodality screening (CA125 testing and transvaginal

ultrasound utilising ROCA versus ultrasound alone versus no screening [5]. No differences in relative mortality reduction was observed between the groups. However a pre-specified analysis of the cohort excluding prevalent cases at diagnosis found a 28% mortality reduction in years 7-14. The trial concluded that longer follow up was required to determine the efficacy and cost effectiveness of screening for EOC. The large Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial performed earlier in the United States also found an apparent mortality benefit in years 7-14 for ovarian cancer screening however no differences were observed at extended follow up (median 15 years) [6]. At present, routine multimodality screening is currently not recommended for the general population.

There is now firm evidence that EOC is made up of five main histological subtypes: high grade serous ovarian carcinoma (HGSOC) (70%), low grade serous ovarian carcinoma (LGSOC)(<5%), endometrioid ovarian carcinoma (EnOC) (10%), clear cell ovarian carcinoma (CCOC)(10%) and mucinous ovarian carcinomas (MuOC) (3%) [7]. Each histological subtype is molecularly diverse and has distinct biology and clinical behaviours. They also differ in their pre-cursor lesions, site of origin, stage of presentation, chemotherapy-responsiveness and prognosis [8]. Despite this, the primary management of all EOCs remains largely identical with aggressive cytoreductive surgery and platinum-based chemotherapy as standard of care. However, it now clear that each histological sub-type warrants an individualised approach in its management.

In the following sections, the general management of EOC is discussed followed by a detailed overview of each histological subtype and the implications of management in each.

1.2 Clinical management

1.2.1 Surgery in early stage disease

The International Federation of Gynecology and Obstetrics (FIGO) stage is a powerful prognostic indicator in EOC. The aim of surgery in early stage disease is not only to resect the tumour but to perform adequate staging. In Europe, comprehensive surgical staging of early stage disease (Stage IA-IIA) involves a midline laparotomy, bilateral salpingo-oopherectomy, hysterectomy, infracolic omentectomy, biopsies of suspicious nodules within the peritoneum and iliac and retro-peritoneal lymph node sampling [9].Bulky lymph nodes should be surgically resected. Comprehensive surgical staging has been shown to upstage up to 30% of patients depending on histological sub-type [10, 11]. It is thus vital in providing accurate prognostic information and influencing the need and type of post-operative treatment.

Whilst lymph node sampling is widely accepted as part of comprehensive surgical staging in early stage disease, systematic aortic and pelvic lymphadenectomy is controversial. Studies have shown up to 24% of apparent early stage patients are upstaged following removal of lymph nodes (i.e. occult stage III disease)[12-15], and retrospective analyses have demonstrated a survival benefit of this procedure[16]. Only one prospective randomised phase III study led by Maggioni et al of systemic lymphadenectomy versus lymph node sampling in early stage EOC has been performed to date [17]. This trial found a greater number of patients with positive lymph nodes in those undergoing lymphadenectomy versus lymph node sampling (22% versus 9%; P=0.007). However only a trend was observed for progression free survival (PFS) and overall survival (OS). This trial was only powered to detect a 10% difference in the frequency of lymph node metastases as it was deemed unfeasible to be powered for survival. Furthermore, lymphadenectomy was associated with greater surgical morbidity. As such, systemic lymphadenectomy in early stage disease is not routinely performed in Europe.

1.2.2 Surgery in advanced stage disease

In addition to stage, the extent of residual disease following surgical cytoreduction in advanced stage disease (stage IIB-IV) is an important independent prognostic indicator in EOC [18, 19]. Aggressive surgical cytoreduction in EOC reduces tumour bulk thus synchronising cell growth, increasing chemotherapy concentrations and reducing the development of resistant clones [19]. Complete macroscopic surgical cytoreduction (0cm) confers the best prognosis, whereas the survival benefit of 1-10mm compared to more than 1cm residual tumour is marginal [18].

The aim of surgery in advanced stage EOC is therefore complete macroscopic cytoreduction. Surgical procedures which may be required to achieve this include peritoneal stripping, bowel and diaphragmatic resection, splenectomy and resection of bulky lymph nodes [2]. Lymphadenectomy with macroscopically normal lymph nodes confers no OS benefit [20]. Occasionally, neoadjuvant chemotherapy (NACT) can be employed to render the disease operable and improve the chances of an R_0 resection. The EORTC 55971 (Neoadjuvant Chemotherapy or Primary Surgery in Stage IIIC or IV Ovarian Cancer) [21] and CHORUS trial (Primary chemotherapy versus primary surgery for newly diagnosed advanced ovarian cancer) [22] were two large randomised phase III trials which showed no

differences in PFS or OS in patients who underwent upfront primary cytoreductive surgery compared to NACT and delayed surgery. Lower rates of peri-operative complications and post-operative deaths were also found in the NACT arm. The main criticism were the short median operating times of less than 3 hours in both trials, suggesting that maximal surgical effort was not employed.

Pre-planned pooled subgroup analyses of both trials did however demonstrate that patients with stage IV disease had both PFS and OS benefit with NACT over primary surgery, whereas stage IIIC patients with lower disease bulk (largest metastatic tumour <5cm) had superior PFS with primary surgery over NACT but no OS benefit [23]. No differences were observed for stage IIIC disease with larger metastases (≥5 cm) [23]. In clinical practice, NACT is reserved for patients with stage IV disease, poor performance status, or in stage III disease in which complete macroscopic cytoreduction is not achievable.

1.2.3 Adjuvant chemotherapy

The two pivotal trials which provide evidence for the use of adjuvant platinum based chemotherapy in early stage EOC are the ICON1 (International Collaborative Ovarian Neoplasm trial 1: a randomized trial of adjuvant chemotherapy in women with early-stage ovarian cancer) [24] and ACTION trials (Impact of Adjuvant Chemotherapy and Surgical Staging in Early-Stage Ovarian Carcinoma: European Organisation for Research and Treatment of Cancer–Adjuvant Chemotherapy in Ovarian Neoplasm Trial) [25]. In both trials which were run in parallel, a total of 925 patients were randomised to receive at least 6 cycles of post-operative platinum based chemotherapy (at least 4 cycles in the ACTION trial) versus observation. Combined survival analyses of both trials showed a significant improvement in recurrence-free survival (76% vs 65%; HR=0.64, P=0.001) and OS at five years (82% vs 74%; HR=0.67, P=0.008) with adjuvant chemotherapy. This effect remained consistent even at ten years with estimated HR of 0.72 for OS and 0.67 for PFS [26].

However, these trials have been criticised for a number of reasons. ICON1 had a broad entry criteria which included patients with both good prognosis as well as patients who did not undergo comprehensive surgical staging. Furthermore, only a third of patients in the ACTION trial had successful surgical staging despite maximum surgical effort [27]. Retrospective analyses of the ACTION trial demonstrated a relapse free survival (RFS) and OS benefit in those who were sub-optimally staged, but not in the optimally staged group. Furthermore, these trials did not distinguish between the histological subtypes of EOC which have diverse chemotherapy sensitivities. In clinical practice, six cycles of platinum-based adjuvant chemotherapy is the recommendation for patients with grade 3, stage IC-IIA disease or CCOC [2].

In advanced stage disease (stage IIB-IV), the addition of paclitaxel to platinum following surgical cytoreduction has been evaluated in four phase III randomised controlled trials. The GOG 111 (Cyclophosphamide and cisplatin compared with paclitaxel and cisplatin in patients with stage III and stage IV ovarian cancer) [28] and OV-10 trials (Randomized Intergroup trial of cisplatin-paclitaxel versus cisplatin-cyclophosphamide in women with advanced epithelial ovarian cancer) [29] demonstrated a survival benefit whereas the GOG 132 (Phase III randomized study of cisplatin versus paclitaxel versus

cisplatin and paclitaxel in patients with suboptimal stage III or IV ovarian cancer: a Gynecologic Oncology Group study) [30] and ICON3 trial (Paclitaxel plus carboplatin versus standard chemotherapy with either single-agent carboplatin or cyclophosphamide, doxorubicin, and cisplatin in women with ovarian cancer: the ICON3 randomised trial) [31] demonstrated no survival differences between platinum chemotherapy versus the platinum/taxane combination. The main reasons for differential outcomes between the 4 trials were due to differences in the control arms, patient entry criteria, doses of cisplatin and paclitaxel used, and a significant proportion of crossover to the experimental arm.

In clinical practice, the use of doublet chemotherapy is considered standard of care worldwide as long as patients are deemed fit enough for treatment. This practice has also been largely extrapolated to early stage disease and the addition of a taxane to a platinum backbone is considered routine in this setting.

1.2.4 Intraperitoneal chemotherapy

As most EOC will relapse within the peritoneal cavity, intraperitoneal (IP) chemotherapy has been investigated as an alternative route of treatment delivery with the aim of delivering higher concentrations of chemotherapy to ovarian tumours, whilst minimising systemic toxicity [32]. When compared to intravenous delivery, IP cisplatin demonstrates a 10-20 fold increase in IP concentration when compared to plasma [33, 34]. Similarly, IP paclitaxel also demonstrated a 1000 fold increase in IP concentration over plasma [35, 36]. Small tumours of less than 5mm as well as avascular tumours are more likely to demonstrate this biological advantage compared to bulky or well-perfused tumours.

The three largest randomised phase III clinical trials performed demonstrated that IP chemotherapy improved OS over IV chemotherapy in patients with advanced EOC following optimal surgical cytoreduction. They were GOG 104 (IV cisplatin 100 mg/m^2/IV cyclophosphamide 600 mg/m^2 versus IP cisplatin 100 mg/m^2/IV cyclophosphamide 600 mg/m^2), GOG 114 (IV cisplatin 75mg/m^2/IV paclitaxel 135mg/m^2 (24 hours) versus IV carboplatin AUC 9 (2 cycles)/IP cisplatin 100mg/m^2 /IV paclitaxel 135mg/m^2 (24 hours)) and GOG 172 (IV cisplatin 75mg/m^2/IV paclitaxel 135mg/m^2 (24 hours) versus IP cisplatin 10 mg/m^2 /IV paclitaxel 135mg/m^2 (24 hours)/IP paclitaxel 60mg/m^2 (day 8)) [37]. In the latest GOG 172 trial, the OS benefit of IP chemotherapy over IV was 66.9 months compared to 49.5months (P=0.0076). These results led to a National Cancer Institute alert in 2006 which reported on a meta-analyses of 7 randomised trials of IP chemotherapy. This showed that IP chemotherapy reduced the risk of death by 21% (HR=0.79 (95%CI 0.70-0.89) and conferred a survival advantage of approximately 12 months.

However, each trial sustained a number of criticisms. In particular, the IP arm of the GOG172 arm was criticised for including a weekly schedule of IP paclitaxel (60mg/m^2 on day 8), and higher doses of IP cisplatin (100mg/m^2) compared to the three weekly schedule of the control arm (IV cisplatin 75mg/m^2 and IV paclitaxel 135mg/m^2 over 24 hours). These variables may have confounded the improvement in OS of IP over IV chemotherapy. In response to this, the GOG 252 trial was the fourth randomised phase III trial comparing three weekly IV carboplatin AUC 6/ weekly IV paclitaxel 80mg/m^2 versus three weekly IP carboplatin AUC 6/weekly IV paclitaxel 80mg/m^2 versus three weekly IV paclitaxel 135 mg/m^2 day 1

(3 hours)/IP cisplatin 75mg/m^2 day 2/IP paclitaxel 60mg/m^2 day 8 [38]. All three arms included a weekly schedule of paclitaxel and included the use of bevacizumab. Notably, the IP dose of cisplatin was reduced to 75mg/m^2 and the infusion time of paclitaxel was reduced to 3 hours. This trial demonstrated no difference in PFS and OS data is still immature.

To date, the role of intraperitoneal chemotherapy in advanced stage disease remains uncertain. Furthermore, IP chemotherapy is associated with greater toxicity, risk of catheter complications and inconvenience to patients. As such, it is not considered standard of care and its use not widely adopted.

1.2.5 Relapsed disease

Up to 80% of patients with advanced stage EOC (most of which are HGSOC) will exhibit high response rates to initial platinum chemotherapy. However, most patients will eventually relapse within 3 years of diagnosis [8]. Further treatment for relapsed disease is only initiated upon symptomatic relapse rather than on the basis of a raised CA125 alone. No survival difference was demonstrated in the MRC OVO5: EORTC 5595 clinical trial when comparing patients who had chemotherapy initiated upon CA125 only asymptomatic relapse versus those with symptomatic relapse [39]. With time, the interval between each treatment gradually shortens with reducing efficacy and greater toxicity. The choice of chemotherapeutic agents in relapsed EOC is based on the platinum-free interval (PFI) which has been shown to be directly proportional to tumour response rates to platinum agents [40]. This has been historically classified as platinum sensitive (PFI greater than 12 months), partially platinum sensitive (PFI 6 to 12 months), platinum resistant (PFI less than 6 months), and platinum refractory (no response to first line platinum chemotherapy), although a recognised continuum exists [41].

1.2.5.1 Platinum sensitive and partially platinum sensitive disease

Patients with platinum sensitive and partially platinum sensitive relapsed disease are often re-challenged with three weekly platinum doublet chemotherapy with the likely-hood of response over 50% in this setting. The ICON4 and AGO-OVAR-2.2 trials which were run in parallel, showed that carboplatin and paclitaxel was superior to single agent carboplatin (hazard ratio 0.82 [95% CI 0.69-0.97], p=0.02) [42]. This corresponded to an improvement in median OS of five months (29 vs 24 months [1-11) with a seven percent improvement at two years (57% versus 50% [95% CI for difference 1-12]) [42]. Of note, over 70% of patients had a PFI of greater than 12 months. The Calypso trial was another randomised phase III trial which showed that carboplatin and pegylated liposomal doxorubicin was non-inferior (HR 0.99; P=0.94), and also demonstrated a PFS benefit (HR 0.82; P=0.005), when compared to carboplatin and paclitaxel [43, 44]. The PFS benefit was also found to be more pronounced in the partially sensitive cohort (HR 0.73). Furthermore, the carboplatin/paclitaxel arm of this trial had higher frequencies of treatment discontinuation due to non-haematological toxicity (15% versus 6%; P<0.001), as well as higher rates of grade 2 to 4 neuro toxicity (26.9% versus 4.9%), compared to the carboplatin/ pegylated liposomal doxorubicin arm. In view of this, carboplatin and pegylated liposomal doxorubicin is thus the preferred agent of choice in platinum sensitive or partially platinum sensitive relapsed disease.

1.2.5.2 Platinum resistant and refractory disease

Patients with platinum resistant or refractory disease are a poor prognostic group with a median OS of approximately 12 months with low response rates to chemotherapy of between 10-15 % [45]. There is little data supporting the optimal sequencing of chemotherapy agents in platinum resistant EOC. The majority of data are derived from early phase (I/II) trials, or from sub-group analysis of platinum resistant cohorts in randomised phase III trials [46-48]. Platinum resistance can be overcome by dose dense scheduling of platinum or taxanes (weekly carboplatin/paclitaxel; weekly paclitaxel; weekly cisplatin/etoposide) [49], and other options include pegylated liposomal doxorubicin and topotecan. In general, single chemotherapeutic agents are used rather than multi-agent chemotherapy as they increase toxicity without additional survival benefit.

Only a handful of randomised phase III studies have been performed specifically in platinum resistant disease alone [45, 50]. The only trial that demonstrated an improvement in PFS was the AURELIA study of investigators' choice of chemotherapy with or without bevacizumab in platinum resistant EOC. Sub-group analysis demonstrated an OS benefit when paclitaxel was used [51] (to be discussed in greater detail in section 1.2.6). As such, the use of paclitaxel with bevacizumab is being increasingly favoured as the first treatment of choice in platinum resistant EOC.

1.2.6 Bevacizumab

The vascular endothelial growth factor (VEGF) is commonly expressed in EOC and is responsible for stimulating angiogenesis, a mechanism of tumour growth and metastases [52]. High VEGF expression and angiogenesis is associated with disease extent and poor survival [53, 54]. Bevacizumab is a humanised monoclonal antibody which targets VEGF and its role in the front line and relapsed settings of EOC has been evaluated in 4 pivotal phase 3 trials.

1.2.6.1 Bevacizumab in first line treatment

Both GOG-0218 [55] and ICON7 [56] were randomised controlled trials evaluating bevacizumab in the first line treatment of patients with advanced EOC (stage III/IV) following maximal primary cytoreductive surgery. In both trials, PFS was the primary end point and post-operative chemotherapy used was 6 cycles of post-operative carboplatin (AUC 6) and paclitaxel (175mg/m^2). In GOG-0218, the 3 trial arms were 1) chemotherapy and placebo, followed by maintenance placebo, 2) chemotherapy and bevacizumab (from cycle 2 to 6) followed by maintenance placebo, and 3) chemotherapy and bevacizumab (from cycle 2 to 6) following by maintenance bevacizumab alone. In ICON7, the 2 trial arms were 1) chemotherapy and placebo (5-6 cycles) followed by maintenance placebo, and 2) chemotherapy and bevacizumab (5-6 cycles) followed by maintenance bevacizumab. In GOG-0218, bevacizumab was delivered at a three weekly dose of 15mg/m^2 for up to 16 cycles in the maintenance period, whereas the dose in ICON7 was lower at 7.5mg/m^2 delivered for up to 12 cycles in the maintenance phase.

In GOG-0218, an overall median PFS benefit (14.1 versus 10.3 months, HR 0.717, p<0.001) was observed [55]. In ICON7, an initial PFS benefit was observed (HR0.81, p=0.04) after 19.4 months [56], but lost significance after 48.9 months follow up (P=0.25) [57]. No differences in OS was observed for

both trials (GOG-0218: P=0.53; ICON 7: P=0.85). However, in ICON7, a pre-defined poor prognosis subgroup of patients (defined as stage IV, inoperable stage III or sub-optimally cyto-reduced (>1cm) stage III) demonstrated a median PFS (15.9 versus 10.5 months, HR 0.68, 95% CI 0.55-0.85; P<0.001) and median OS benefit (39.7 month versus 30.3 months, HR0.78, CI 0.63-0.97, P=0.01) in those who received bevacizumab over placebo [56, 57]. A subsequent unplanned analysis of stage IV patients in the GOG-0218 study also demonstrated an OS benefit in those who received maintenance bevacizumab over placebo (HR0.72, CI 0.53-0.97) [58], supporting its front line use in poor prognostic patients as defined in ICON7. These findings also support the biological need for angiogenesis in proliferating tumours for efficacy to be demonstrated. Further to this, several studies have been performed exploring the differential impact of bevacizumab on molecular subtypes of HGSOC [59, 60].

1.2.6.2 Bevacizumab in relapsed disease

The OCEANS trial was a phase III clinical trial which recruited patients with platinum sensitive EOC who were randomised to receive carboplatin (AUC4), gemcitabine (1000mg/m2) alone or with bevacizumab (15mg/m2) until disease progression or toxicity [61]. The primary endpoint of the trial, PFS, was found to be significantly longer in those who received bevacizumab over those who received placebo (12.4 versus 8.4 months, HR 0.484, P<0.0001). There was no difference in OS [62].

The AURELIA trial recruited patients with platinum resistant EOC who were randomised to receive chemotherapy of investigators choice (pegylated liposomal doxorubicin, weekly paclitaxel, or topotecan) alone, or with bevacizumab (10mg/kg 2 weekly with chemotherapy, or 15mg/kg three weekly during the maintenance phase) until disease progression or toxicity [45]. Median PFS was improved in the bevacizumab group over placebo (6.7 versus 3.4 months, HR0.48, P<0.001), no OS benefit was seen although it is worth noting 40% of patients in the placebo group crossed over to receive bevacizumab. An exploratory analysis found a greater treatment effect on PFS (median 10.4 versus 3.8 months; HR 0.46; 95%CI 0.30-0.71) and OS (median 22.4 versus 13.2 months; HR 0.65; 95% CI, 0.42 to 1.02) observed in those who received paclitaxel together with bevacizumab [51]. It is however worth noting that patients were stratified but not randomised by physician's choice of chemotherapy. Weekly paclitaxel also demonstrates an anti-angiogenic effect which has been shown to be due to down regulation of VEGF as well as its tendency to accumulate in endothelial cells [63]. These findings from the AURELIA trial may thus be due to enhanced angiogenesis and synergy with this combination. Furthermore, a pre-specified analyses in the AURELIA trials found the use of bevacizumab reduced the incidence of paracentesis with each cycle compared to the use of chemotherapy alone. The use of bevacizumab is thus recommended in patients with platinum resistant disease with paclitaxel as the preferred choice of chemotherapy backbone. Patients with recurrent ascites are thus more likely to benefit from this combination.

1.2.7 Secondary and tertiary cytoreductive surgery

There is emerging evidence to support the role of secondary cytoreductive surgery for relapsed disease in well-selected patients.

The DESKTOP I study was a retrospective analysis of 267 patients with platinum sensitive relapsed EOC who had undergone secondary cytoreductive surgery [64]. Complete surgical cytoreduction was the only factor associated with a significant improvement in OS compared with post-operative residual disease (45.2 vs 19.7 months; HR 3.71; $p<0.0001$). This study also derived the German Gynaecological Oncology Working Group (AGO) score to help predict resectability. Components of the score were previous complete surgical resection, good performance status and the absence of ascites.

The DESKTOP II study conducted prospective validation of the AGO score in platinum sensitive relapsed ovarian cancer patients [65]. It found that more than two thirds of patients at first relapse with a positive AGO score achieved complete cytoreduction. The study also evaluated the safety and feasibility of surgery in these patients with an 11% rate of second surgery and 0.8% peri-operative mortality rate.

Two phase III randomised trials have been performed to evaluate the role of secondary cytoreductive surgery. DESKTOP III compared cytoreductive surgery followed by platinum chemotherapy versus chemotherapy alone in patients with platinum sensitive relapsed EOC with a positive AGO score [66]. This trial showed a 5.6 month PFS benefit (19.6 versus 14 months; P<0.001) and 7.1 months improvement in time to next therapy (21 versus 13.9 months; P<0.001) in those who had complete secondary surgical cytoreduction. OS data is currently immature. In the USA, the GOG-0213 trial is another phase III trial in platinum sensitive relapsed EOC with a two part randomisation [67]. The first evaluates the role of concurrent and maintenance bevacizumab compared to platinum-doublet chemotherapy alone, and second part explores the role of secondary cytoreductive surgery. In the latter, patients with investigator-determined resectable disease were randomised as per the DESKTOP III trial. No differences in PFS and OS were observed in the whole cohort and subgroup analyses are currently awaited.

At present, the results of the DESKTOP III trial support the use of secondary cytoreductive surgery in well-selected patients in order to improve PFS. The final OS results will help determine its role as a standard of care.

1.2.8 Endocrine therapy

This is discussed in detail in section 1.3.

1.2.9 PARP inhibitors

The role of poly (adenosine diphosphate) ribose polymerase (PARP) inhibitors is discussed in section 1.4.1.1.

1.3 Hormones and ovarian cancer

Epidemiological studies have shown that steroid hormones are intrinsically linked to the development and promotion of EOC [68]. Hormonal risk factors include the use of hormone replacement therapy (HRT) and nulliparity [69], whereas the use of the oral contraceptive pill [70, 71], tubal ligation [72], and younger age at pregnancy have been shown to be protective [73]. Patients with a hyper-androgenic state, such as those with polycystic ovarian syndrome [74] and truncal obesity [75], have also been found to have a higher incidence of EOC.

In particular, the association of HRT use in post-menopausal women and the development of EOC has been extensively investigated. A meta-analysis of 52 epidemiological studies which included 12110 women was performed by the Collaborative Group on Epidemiological Studies of Ovarian Cancer demonstrated a number of findings [69]. It found that the risk of EOC was greater in those who had ever used HRT (current or recent use) compared to never users. This was highest for serous (HR1.53, $p<0.0001$)) and EnOC (HR1.42; $p<0.0001$) in current or recent users of HRT, with no effect on CCOC and MuOC. Interestingly, although the risk of developing EOC declined with time, the risk remained even after ten years following cessation of HRT. In a similar large scale analysis of 23257 women, the duration of oral contraceptive use correlated with the magnitude of EOC risk reduction ($P<0.0001$) [70]. This beneficial effect was observed on all histological subtypes of EOC apart from MuOC. Interestingly, this risk reduction persisted for more than 30 years but the effect was attenuated over time.

1.3.1 Hormone receptor expression in EOC

Most EOC express hormone receptors, of which ER and PR have been most studied. ER is made up of two isoforms, ER-alpha (ERα) and ER-beta (ERβ). ERα is a transcription factor which is ligand-activated and regulates gene expression in response to oestrogen and other extra-cellular signals [76]. It is the isoform most clearly linked to the endocrine responsiveness in EOC (discussed in section 1.3.2). The role of ERβ is however less well understood. It is expressed at high levels on the surface of normal ovarian cells, whilst reduced expression is found in EOC [77] with even lower levels found in metastasises [78]. This observation suggests that ERβ may play a tumour suppressor role [79]. PR expression is regulated by the ERα gene, and is a surrogate for a functionally intact ERα pathway [80]. In contrast to ERα, activation of PR inhibits cell proliferation. In breast cancer, PR activation changes the pattern of ERα chromatin binding, which result in the increased expression of anti-proliferative genes [80]. Similarly in ovarian cancer, activation of PR has been shown to induce cell apoptosis.

The expression of ERα (referred to as ER from this point) and PR vary across the histological subtypes of EOC (Table 1). The Ovarian Tumour Tissue Analysis consortium study was the largest study of 3000 women to evaluate this [81]. A three-tier scoring system was used: negative (<1% nuclear stain), weak (1-50% nuclear stain), and strong (≥50% nuclear stain). Strong or weak ER expression was highest in HGSOC (80.7%; 60.2% strong, 20.5% weak), LGSOC (87.6%; 71.2% strong, 16.4% weak), and EnOC (76.6%; 60.2% strong, 16.4% weak), with lowest levels in MuOC (15.7% strong, 5.1% weak) and CCOC (13.7% strong; 5.8% weak). In contrast, strong or weak PR staining was highest for EnOC (67.4%; 44.4% strong, 23.0% weak), LGSOC (57·4%; 32.7% strong, 24.8% weak), moderate for HGSOC

(31·1%; 7.5% strong, 23.7% weak), and lowest for MuOC (16·4%; 8.7% strong, 7.7% weak) and CCOC (8·0%; 3.0% strong, 5.0% weak).

Co-expression of ER and PR was most common in EnOC (81.6%), LGSOC (61.9%) and MuOC (63.2%), and lowest for HGSOC (34.3%) and CCOC (32.4%). Other studies which distinguish between histological subtypes also support this data [82, 83].

Table 1: ER and PR expression in histological subtypes of epithelial ovarian carcinomas in the Ovarian Tumour Tissue Analysis consortium study [81].

Type	ER			PR		
	Negative	Strong	Weak	Negative	Strong	Weak
High grade serous	19%	60%	21%	69%	7%	24%
Low grade serous	13%	71%	16%	43%	33%	25%
Endometrioid	23%	60%	16%	33%	44%	23%
Clear cell	81%	14%	6%	92%	3%	5%
Mucinous	79%	16%	5%	84%;	9%	8%

Legend: Strong = ≥50% nuclear stain; Weak = 1-50% nuclear stain; ER=oestrogen receptor; PR=progesterone receptor.

The prognostic role of ER and PR has been evaluated in several heterogeneous studies with conflicting results (Table 2). Most of these trials included all histological subtypes, and in particular, did not differentiate between HGSOC and LGSOC which have vastly different biology and prognosis [84]. They also used different methods and thresholds in defining receptor positivity, factors which may limit the interpretation of these studies. Meta-analysis of 23 studies performed by Zhaojun et el found PR but not ER was prognostic in EOC [85]. This finding is akin to ER positive breast cancers where high PR expression is associated with more indolent disease, whereas PR negative tumours are more biologically aggressive [86].

In the Ovarian Tumour Tissue Analysis, the prognostic value of ER and PR differed across histological subtypes [81]. Strong PR but not weak PR or any ER expression (strong or weak) was independently associated with survival in HGSOC. In contrast, both strong and weak ER and PR expression were independently prognostic in EnOC. There were no differences for LGSOC, MuOC or CCOC.

Table 2: Studies evaluating ER and PR as a prognostic marker in epithelial ovarian carcinoma.

Study	N	Pathology	IHC threshold	Independent association with OS
Lee et al 2005[87]	322	76.7% serous	>10%	PR but not ER.
Høgdall et al 2007[88]	582	57% serous	≥10%	ER alone, PR alone, and in combination.
Liu et al 2009[89]	148	100% G3 serous	≥1%	Neither ER nor PR. Trend for OS observed for age ≤ 5five years.
Nodin et al 2010[90]	154	58.4% serous	>10%	Neither ER or PR.
Burges et al 2010[91]	100	100% serous	≥10%	ER alone but not PR.
Lenhard et al 2012[92]	155	70.5% serous	>10% with immunoscore >2	PRβ but not ER.
Sieh et al 2013[81]	2933	59.4% HGSOC	<1%; 1-50%; ≥50%	Strong PR in HGSOC, any ER and PR in EnOC, no differences for LGSOC, MuOC, or CCOC.
Matsuo et al 2014[93]	221	100% HGSOC	≥ 5% with stain intensity of ≥ 1 +	Neither ER or PR.
De Toledo et al 2014[94]	152	44.1% serous	Allred score≥4	Neither ER or PR.
Jonsson et al 2015[83]	118	Serous (74%) and EnOC (26%)	≥10%	PR but not ER.
van Kruchten et al 2015[95]	196	70% serous	>10% with immunoscore >2	Neither ER or PR.
Feng et al 2016[96]	875	100% HGSOC	>10%	PR but not ER.

Legend: G=grade; N=number; IHC=immunohistochemistry; HGSOC=high grade serous ovarian carcinoma; EnOC=endometrioid ovarian carcinoma; LGSOC=low grade serous ovarian carcinoma; MuOC=mucinous ovarian carcinoma; CCOC=clear cell ovarian carcinoma; ER=oestrogen receptor; PR=progesterone receptor; OS=overall survival.

In contrast to ER and PR, the androgen receptor (AR) has been less researched. AR plays a role in promoting cell proliferation and reducing cell death [97]. In its basal inactive state, AR is bound to heat shock protein and other cellular components [98]. Androgens actives AR which triggers downstream cell signalling events which include dissociation from the heat shock protein, phosphorylation and dimerization [98]. This culminates in nuclear translocation of AR which binds to targeted DNA sequences, known as androgen response elements, as well as other co-factors [98]. This AR complex can then regulate gene expression of the cell through targeted gene transcription [99]. In ovarian cancer cell lines, androgens has been shown to promote motility and invasion, suggesting that AR activation may result in a more aggressive ovarian cancer phenotype [100].

AR expression in EOC has been mainly evaluated in small studies with frequencies between 43.5-86% [90, 99, 101-103]. Studies have shown that AR is differentially expressed across histological subtypes [99]. In general, higher expression was found in serous tumours compared to non-serous tumours, however the distinction between HGSOC and LGSOC was often not made [87, 94, 102]. Another large study of 876 HGSOC demonstrated AR expression in 35.6% of tumours. Furthermore, no association between AR expression and FIGO stage was demonstrated [96].

The prognostic value of AR has been met with conflicting results (Table 3). Similar to ER and PR, this is likely due to heterogeneity of the studies with respect to histological subtype and methods and thresholds used to define AR positivity. The largest of these studies performed in one histological subtype was by Feng et al of 875 HGSOC [96]. Here, AR positivity (>10% of tumour cells stained) was independently associated with OS. Interestingly, tumours which were AR positive but ER and PR negative demonstrated superior survival compared to other combinations of hormone receptor positivity. Similar conclusions regarding the independent prognostic value of AR in HGSOC were made in Martins et al [104].

Table 3: Studies evaluating AR as a prognostic biomarker in epithelial ovarian carcinoma.

Study	N	IHC threshold	Pathology	Independent association with survival
Lee et al 2005[87]	322	>10%	76.7% serous	No
De Toledo et al 2014[94]	152	Allred score≥4	44.1% serous	No
Van Kruchten et al 2015[95]	121	≥ 10%	70% serous	No
Nodin et al 2010[90]	154	>10%	58.4% serous	Prolonged DSS in serous tumours
Martins et al 2014[104]	255	Low AR=1-49%; high AR= ≥50%	100% HGSOC	Low AR associated with shorter OS
Jonsson et al 2015[83]	118	≥ 10%	Serous (74%) and EnOC (26%)	AR expression alone, and co-expression with PR, was associated with five year PFS and OS
Feng et al 2016[96]	875	>10%	100% HGSOC	AR associated with OS
Gomora et al 2018[105]	81	Histoscore>30	36% HGSOC	

Legend: IHC=immunohistochemistry; EOC=epithelial ovarian carcinoma; HGSOC=high grade serous ovarian carcinoma; AR=androgen receptor; PR=progesterone receptor; PFS=progression free survival; DSS=disease specific survival; OS=overall survival.

1.3.2 Endocrine sensitivity of EOC

The degree of ERα expression mediates the response to endocrine therapy in EOC. The first pre-clinical studies of endocrine therapy in ovarian cancer demonstrated a proliferative effect on ERα positive ovarian cancer cell lines by oestrogen and the anti-mitogenic effect of tamoxifen in these cell lines [106, 107]. Conversely, there was no effect of E2 on ERα negative or ERβ positive cell lines. ERα induced gene expression changes observed in these ovarian cancer cell lines have also been shown to be blocked by the tamoxifen [108]. In a Japanese study, treatment with aromatase inhibitor demonstrated an improvement in survival of strongly ER-alpha positive OVCAR-3 human ovarian cancer cells compared to weakly ER positive ovarian cancer cells in xenograft models [109]. This improvement in survival was associated with an improvement in angiogenesis and ascites in the strongly ER positive xenografted mice.

The role of endocrine therapy in EOC has been investigated in over 50 phase II clinical trials. These trials of investigating different endocrine therapy agents used were highly heterogeneous. They included all histological subtypes and were mostly performed in heavily pre-treated patients. In particular, most were un-selected for ER status and in those who were, different ER measurement thresholds were used to define receptor positivity. As such, low response rates of 0% to 18% and moderate disease stabilisation rates of 20% to 50% have been observed (Table 4). Furthermore, no survival benefit was demonstrated in the only phase III randomised clinical trial performed of tamoxifen versus chemotherapy in patients with platinum resistant EOC [110]. The use of endocrine therapy is thus not a standard of care with variable practice worldwide.

Table 4: A selection of trials evaluating endocrine therapy in epithelial ovarian carcinoma.										
Trial	**ET**	**N**	**Path**	**Thres-hold**	**ER+**	**≥ 2 lines of chemo**	**Response**			
							Crit.	**ORR**	**SD**	**CBR**
Hatch 1991 [111]	Tam	105	54% serous	fmol/mg	93%>0 fmol/mg	100%	RECIST	18%	38%	56%
Bowman 2002 [112]	Let	60	43% serous	H-score ≥1	97%	50%	Ca125	8%	24%	32%
							UICC	0%	20%	20%
Smyth 2007 [113]	Let	42	52% serous	H-score ≥150	100%	46%	Ca125	17%	26%	43%
							RECIST	9%	42%	51%
Argenta 2009 [114]	Fulv	26	62% serous	Nuclear stain ≥ 10%	100%	100%	Ca125	UK	UK	UK
							RECIST	0%	50%	50%
Del Carmen 2003 [115]	Anast	53	Mullerian	Nuclear stain ≥ 1%	71%	72%	RECIST	2%	42%	44%
Papadimitriou 2003 [116]	Let	27	UK	Nuclear stain ≥ 1%	73%	41%	RECIST	15%	19%	34%
Bona-ventura 2017 [117]	Anast	49	High grade EOC	Nuclear stain ≥ 10%	100%	All platinum resistant or refractory	RECIST	0%	0%	27%

Legend: ET= endocrine therapy; N=number; Path=pathology; ER+=oestrogen receptor positive; Crit. = criteria; ORR=objective response rate; SD=stable disease; CBR=clinical benefit rate; RECIST=response evaluation criteria in solid tumours; UICC= union for international cancer control; H-score=histoscore; Tam=tamoxifen; Let=letrozole; Fulv=fulvestrant; Anast=anastrazole; UK=unknown; EOC=epithelial ovarian carcinoma.

However, there is now emerging retrospective data to support the use of endocrine therapy specifically in LGSOC in both the relapsed [118] and first-line maintenance settings [119]. The first and largest retrospective study was performed by the MD Anderson group of 64 women with relapsed LGSOC who received a total of 89 separate endocrine regimens [118]. There was an ORR of 9% (2 CR, and 6 PR) with a disease stabilisation rate of 62%, amounting to a CBR (CR+PR+SD) of 71%. 61% of patients remained on endocrine therapy at least six months or more. The overall median time to progression was 7.4 months (95% CI 6.0-8.9).

This study prompted a further retrospective analysis by the MD Anderson group evaluating the role of maintenance endocrine therapy in the first line management of LGSOC [119]. Inclusion criteria were patients with stage II to IV LGOSC who had an attempt at primary cytoreductive surgery followed by platinum based chemotherapy and with sufficient clinical information at follow up. Between 1981 and 2013, 203 patients were included in this study. 74% of patients had no residual disease and 26% had residual disease following adjuvant chemotherapy. Patients who received maintenance endocrine therapy within 3 months of completing post-operative chemotherapy (n=70; 54% letrozole, 29% tamoxifen, 3% anastrazole, 6% leuprolide acetate) were compared to those who underwent observation (n=133).

The median duration of endocrine therapy was 33.3 months (range 1-223 months). There was a significantly improved median PFS in patients who received maintenance endocrine therapy compared to the observation group of 64.9 months versus 26.4 months ($p<0.001$), respectively. The effect was more marked in patients who were disease free post chemotherapy as compared to those who had persistent disease with a PFS of 81.1 months versus 30.0 months ($p<0.001$) and 38.1 months versus 15.2 months ($p<0.001$), respectively. In the multivariable cox proportional regression analysis, patients who received maintenance endocrine therapy were less likely to experience disease progression as compared to those who did not (HR 0.44; 95% CI, 0.31 to 0.63, $p<0.001$).

Although there were no differences in OS between the two groups, there were significant differences when patients were stratified for residual disease post chemotherapy. The median OS in those with no residual disease and residual disease post chemotherapy was 191.3 months versus 106.8 months and 93.3 months versus 44.4 months ($p=0.014$), respectively. These two pivotal studies have demonstrated that the use of endocrine therapy plays a role in the management of LGSOC in both the relapse and maintenance settings. In response to this data, prospective clinical trials evaluating the role of maintenance letrozole in LGSOC are currently being designed.

Similar data is emerging in HGSOC. Heinzelmann-Schwarz et al was a retrospective study which compared the use of maintenance letrozole versus observation in 50 patients with HGSOC [120]. Here, patients who received maintenance letrozole had a significantly longer RFS compared to the observation group. At 24 months, 60% of the letrozole group were relapse free compared to 38.5% in the control group ($P=0.035$). This effect was also maintained in the small cohort who received concurrent bevacizumab for residual disease. At 12 months, 87.5% of the letrozole group was relapse free compared to 20.8% of the control cohort ($P=0.026$). The authors conclude that this data is

hypothesis generating and that prospective clinical trials of maintenance letrozole in HGSOC are warranted. The studies performed by Gershenson et al and Heinzelmann-Schwarz et al illustrate the importance of evaluating the role of endocrine therapy in histological sub-type specific clinical trials.

There has also been interest in the use of androgen blockade in EOC as studies have found AR expression to be a biological marker for androgen sensitivity [121]. The CORAL phase II trial evaluated abiraterone in AR positive EOC. 83% of this study cohort was HGSOC and 47% of patients received 3 or more prior lines of therapy [122]. Only one patient (2%) had a response to therapy which occurred in an AR positive LGSOC with a duration of therapy of 47 weeks. In the AR positive cohort (>10% staining), there was a prolonged period of disease control of 6 months or greater in 14% of patients. Unfortunately, this clinical trial was halted due to low responses seen in this trial.

1.4 Histological subtypes of epithelial ovarian cancer

The management of EOC has started to account for biological differences of each subtype. Here, the different clinical and molecular differences between each subtype and how this is being used to guide treatment strategies are discussed.

1.4.1 High grade serous carcinoma

The majority of patients with EOC are diagnosed with HGSOC (70%) [8]. These are biologically aggressive tumours which commonly present with advanced staged (stage III/IV) disease. They display exquisite sensitivity to platinum-based chemotherapy with response rates in the region of 80% in the first line setting [8]. However despite this, most patients will recur within the first three years of diagnosis and ultimately die of their disease.

Contrary to its name, most HGSOC originate from the fimbrial end of the fallopian tube with serous tubal intra-epithelial carcinoma (STIC) as its precursor lesion [123, 124]. This was first discovered in patients with germline *BRCA* mutations who underwent prophylactic salpingo-oopherectomy [125-128]. Pathologically, HGSOC also display a myriad of architectural variants, including papillary, nested, cribriform, glandular, solid and single cells. It displays marked atypia with greater than 12 mitoses per 10 high power fields, and typically expresses WT1, p53, MIB1, Her-2 neu, c-kit, bcl-2 and p16 on immunohistochemistry [129].

It is molecularly characterised by ubiquitous *TP53* mutations [130], which are thought to be an early event in its pathogenesis [124]. It is also one of the most chromosomally unstable tumours with extensive copy number changes and loss of heterozygosity, as well as defects in DNA repair pathways [131], sharing molecular similarities to basal-like breast cancer [132]. Around half of all HGSOC display homologous recombination repair (HRR) deficiency [131], a crucial repair mechanism of double strand DNA breaks, due to mutations in key HRR genes. Of these, germline mutations in *BRCA1* and *BRCA2 (gBRCA1/2)* comprise 15% of HGSOC, with another 5-10% due to somatic *BRCA* (s*BRCA*1/2) mutations and epigenetic modifications such as silencing of the *BRCA* protein through *BRCA1* methylation [131]. The remaining mutations in HRR genes include *EMSY* (8% amplification), *PTEN* (7% loss), *RAD51C* (3% hypermethylated), *ATM, ATR, CHEK1 and CHEK2* (2%) and fanconi anaemia genes (5%) [131]. 30% of the remaining non-HRR deficient HGSOC have *CCNE1* amplification which has been found to confers platinum resistance and a poorer prognosis [133, 134]. Other significantly mutated genes include *RB1, NF1, FAT3, CSMD3, CABRA6* and *CSK12* [131] . Recently, comprehensive copy number profiling of 117 HGSOC was performed as part of the BRITROC study, followed by validation in another 527 cases [135]. This study found distinct copy number signatures to predict for the likelihood of platinum resistant relapse and OS.

1.4.1.2 *BRCA* mutated HGSOC and implications of management

Patients with *gBRCA*1/2 mutations are most commonly diagnosed with HGSOC, whilst associations with EnOC and CCOC are very rare [136]. These patients have a cumulative lifetime risk of other malignancies, most commonly breast cancer (65% *BRCA1*, 45% *BRCA2*). Besides the familial implications of this diagnosis, patients with *gBRCA1/2* mutated HGSOC also display distinct clinical characteristics and treatment responses. When compared to *gBRCA1/2* wild-type patients, they are more likely to present at a younger age with a higher frequency of visceral metastases [137]. They also display superior platinum sensitivity in the first line setting and at subsequent relapses [138]. This is further supported by studies which demonstrate that reversion mutations of *BRCA1/2* and other HRR genes, thus restoring HRR, are associated with platinum resistance [139]. Furthermore, patients with *gBRCA1/2* HGSOC demonstrate longer platinum free intervals and have superior OS [140]. This survival advantage is maintained out to ten years in patients with g*BRCA*1 mutations, however becomes less marked and eventually reverses for g*BRCA*2 [141].

BRCA1 and BRCA2 are key proteins involved in repairing double strand DNA breaks through the HRR pathway. As discussed in section 1.4.1, mutations in g*BRCA1/2* as well as other HRR genes render EOC HRR deficient. PARP inhibitors exploit this characteristic by inhibiting single-strand DNA repair. As a result, *gBRCA*-mutated ovarian cancer cells are forced to utilise non-homologous end joining repair pathways which are error prone resulting in cell death caused by synthetic lethality [142]. *gBRCA*-mutated normal cells are however still able to utilise the HRR pathway to repair double strand DNA breaks even if the PARP 1 and 2 proteins are inhibited. This differential effect results in *gBRCA*-mutated ovarian cancer cells being 1000 times more sensitive to PARP inhibitors as compared to *gBRCA*-mutated normal cells [142].

Olaparib was the first PARP inhibitor developed. Early phase clinical trials demonstrated striking single agent activity in just over a third of *gBRCA1/2* mutated heavily pre-treated patients with EOC [143, 144]. A correlation was found between platinum sensitivity and the extent of response to olaparib. In the pivotal phase II trial Study 19, patients who were unselected for g*BRCA*1/2 mutation status with platinum-sensitive relapsed HGSOC who had responded to chemotherapy were randomised to receive maintenance olaparib, or placebo [145]. Patients who received olaparib had a significantly longer median PFS over those received placebo (8.4 versus 4.8 months; HR 0.35; p<0.001). The treatment effect was greater in *gBRCA1/2*-mutated patients (median PFS 11.2 versus 4.3 months, HR 0.18; p<0.0001), although was still maintained but less marked in the g*BRCA1/2* wild-type population (HR 0.54; P=0.0075). Interestingly, 11% remained on drug for over 6 years [146].

This prompted the design of two confirmatory studies, the SOLO1 and SOLO2 trials, which evaluated the role of maintenance olaparib in both somatic and g*BRCA1/2*-mutated ovarian cancer patients in the first- and second-line settings, respectively. In SOLO2, the olaparib group had a significantly longer median PFS of 19.1 months compared to 5.5 months in the placebo group (HR0.3, p<0.0002) [147]. An improvement in median OS of 12.9 months was also observed in those who received olaparib (HR 0.74). In SOLO1, two years of maintenance olaparib in the first line setting demonstrated a superior

PFS with a median of nearly three years over the placebo group (HR 0.3, p<0.001) [148]. The survival curves remained separated at three years suggesting ongoing response despite cessation of the drug, however OS data is currently awaited. Olaparib is now licensed as maintenance therapy in both the first line and relapsed settings in somatic and g*BRCA1/2*-mutated ovarian cancers.

It is also recognised that non-*gBRCA1/2* mutated HGSOC that are HRR deficient also display a similar phenotype to that of *gBRCA1/2* mutant tumours. This phenotype, termed '*BRCA*-ness', has led to interest in identifying additional molecular sub-groups of HRR deficient HGSOC which may also benefit from PARP inhibitors. Phase III clinical trials of other PARP inhibitors, namely rucaparib [149], and niraparib [150], have been performed to investigate this. The NOVA trial randomised both *gBRCA1/2* mutated and non-*gBRCA1/2* mutated HGSOC to receive maintenance niraparib versus placebo following a response to platinum chemotherapy in platinum sensitive relapsed EOC [150]. Patients who received niraparib had a longer PFS then the placebo group. Patients with both *gBRCA1/2* mutated (HR 0.27, 95%CI 0.17-0.41; P<0.001) and non-*gBRCA1/2* mutated EOC (HR 0.45; 95% CI, 0.34 to 0.61; P<0.001) demonstrated a PFS benefit. Within the non-*gBRCA1/2* mutated group, HRR deficient tumours sustained a greater benefit from niraparib (HR 0.38; 95% CI, 0.24 to 0.59, p<0.001). Similar results were observed in the ARIEL3 trial of maintenance rucaparib versus placebo in the platinum sensitive relapsed setting following a response to platinum based chemotherapy [149]. Both niraparib and rucaparib are now licensed for maintenance use in platinum sensitive recurrent HGSOC following a response to platinum chemotherapy regardless of *gBRCA* mutation status. Most recently, both niraparib [151] and olaparib [152] have demonstrated improvements in PFS over placebo when used as first line maintenance therapy in patients with advanced stage HGSOC following surgery and response to platinum-based chemotherapy in an 'all-comer' population and its licensed use in the first line setting is currently awaited.

The current landscape of PARP inhibitor trials are investigating how to enhance the efficacy and overcome resistance to PARP inhibition. There is also considerable interest in the use of immune checkpoint inhibitors in combination with PARP inhibitors. HRR deficient tumours have been shown to have greater neo-antigens and tumour infiltrating lymphocytes compared to HRR proficient tumours [153, 154]. The expression of programmed cell death 1 ligand 1 (PDL-1) has also been shown to be upregulated by PARP inhibitors [155], thus supporting the therapeutic use of this treatment combination.

1.4.2 Low grade serous carcinoma

LGSOC make up 10% of all EOC. It has only emerged as its own disease entity in the last decade following a revision of the original Silverberg three-tiered grading system [156] for serous carcinomas to the two-tiered grading system led by Malpica et al [157]. This grading system which relies on nuclear atypia and mitotic rate, has been shown to be reproducible with minimal inter- and intra-observer variation [158]. The ensuing research has demonstrated HGSOC and LGSOC to be distinct diseases in terms of its pre-cursor lesions, molecular alterations, treatment responses and patient outcomes [84].

In contrast to HGSOC, LGSOC are associated with younger age of diagnosis and poor platinum sensitivity with response rates of 5 % [159-161]. Although it is associated with prolonged survival (median OS of 82 months), the majority of women unfortunately still die of their disease [84]. LGSOC is thought to arise either de novo or from serous borderline tumours (SBT) [162]. This is supported by gene expression profiling which has found LGSOC to cluster together with SBT, but separately from HGSOC [163, 164]. In contrast to HGSOC, LGSOC have been found to be genomically stable [165] and usually exhibit p53 wild-type expression on immunohistochemistry (IHC). In addition, they have higher expression of ER, PR [166] and PAX2 [167] as well as over expression of anterior gradient homolog 3 and insulin-like growth factor 1[168] when compared to HGSOC.

Activating mutations in the mitogen-activated protein kinase (MAPK) pathway are common in the pathogenesis of LGSOC. 20-40% have *KRAS* mutations whilst *BRAF* mutations occur in five percent of cases [84]. *NRAS* mutations have been reported in 9-15% of LGSOC with adjacent SBTs as compared to 0% in SBT [169], postulating this as a possible driver mutation in the pathogenesis of LGSOC. In view of the cumulating data supporting activation of the MAPK pathway in LGSOC, clinical trials of mitogen-activated protein kinase enzyme (MEK) inhibitors have been performed [170]. Recently, the MEK inhibitor trametinib has been found to improve response rates and PFS over physician's choice of therapy (letrozole, tamoxifen, weekly paclitaxel, pegylated lipo-doxorubicin, topotecan) in relapsed LGSOC (PFS: 13.0 vs 7.2 months; HR 0.48; 95% CI, 0.36-0.64; P < .0001) [171], though OS data is awaited. There is also retrospective data to support the use of ET both as maintenance therapy or treatment of relapsed LGSOC and is now commonly used as the first therapy of choice in relapsed disease given is low toxicity profile [118, 119]. The value of adjuvant chemotherapy in this subtype is also starting to be questioned and phase III trials comparing adjuvant ET to platinum based chemotherapy are being planned [172].

1.4.3 Clear cell carcinoma

CCOC make up 10% of EOC and are most prevalent in the Japanese population with an incidence of nearly 25% [173]. Histologically, they are made up of cells with prominent cell membranes and abundant clear cytoplasm, with hobnail cells as a common feature [174]. They also display characteristic morphology consisting of a mix of glandular, tubulocystic, solid and papillary architecture [129]. Although CCOC display a low mitotic rate and are usually well differentiated, they are recommended an assignment of grade 3 [129]. Pathologically, they are important to distinguish from variants of HGSOC such as HGSOC with clear cell change [175]. The majority of CCOC also arise from endometrioisis and display an IHC profile that is WT1 negative, p53 wild-type, and ER negative[173]. Napsin A is also a sensitive and specific marker for CCOC [176].

Molecularly, CCOC are characterised by frequent *ARID1A (50%)* and *PIK3CA (33%)* mutations [177]. MET amplification occurs in 20% of cases and are associated with poor prognosis [178]. Other mutated genes include *KRAS* and *PPP2I1A* [177]. Clinically, they are distinct from HGSOC and most commonly present in younger women with early stage disease [179] Approximately 7% of tumours displaying loss of mismatch repair (MMR) proteins on IHC with a proportion of these related to Lynch Syndrome (discussed in section1.5.6.7) [180]. When adjusted for stage, CCOC have a poorer survival when compared to HGSOC particularly in advanced stage disease. It is considered an intrinsically platinum resistant subtype with response rates of between 11%-27%[181-183], as such the role of adjuvant chemotherapy, particularly in stage I disease, is less certain[182, 184]. Notably, the five year DFS for stage IA and stage IC (surgical rupture alone) CCOC have been found to be comparable and the omission of adjuvant chemotherapy is increasingly being discussed as an option with these patients [184]. Patterns of relapse also differ with pelvic recurrences being most common [185].

In early stage disease, exploratory analysis of the GOG157 trial suggests that only patients with serous tumours benefited from six versus three cycles of chemotherapy whereas there were no additional benefit for CCOC [186]. Given the propensity for pelvic recurrences, pelvic irradiation has also been evaluated as a local treatment following adjuvant chemotherapy which has shown a 20% five year disease free survival benefit in patients with stage IC (surface involvement and positive cytology) and stage II disease in a retrospective study by Hoskins et al [184]. Relapsed CCOC is challenging to treat due to low chemotherapy response rates and poor prognosis. It also shares biological features with renal clear cell carcinoma which include upregulation of anti-angiogenic pathways. As such, clinical trials of antiangiogenic tyrosine kinase inhibitors in CCOC are currently being performed [187-189]. A proportion of CCOC with microsatellite instability (MSI) display increased PD-1/PD-L1 expression which make this subtype attractive candidates for immune checkpoint inhibitors [190]. This is supported by data from early phase immunotherapy studies performed in EOC [191, 192]. In these studies, patients with CCOC sustained complete or partial responses to immune check point inhibitors. Several phase II clinical trials of immune check point inhibitors in CCOC are ongoing [193].

1.4.4 Mucinous carcinoma

MuOC are a rare subtype comprising 3% of EOC. They can be difficult to distinguish between primary MuOC and mucinous carcinomas which have metastasized to the ovary [194]. Colorectal, appendiceal, pancreatic, biliary tract, stomach and cervical cancers are the most common primary sites. Specific IHC patterns involving CK7, CK20, CEA, CA19.9, CA125, ER, CDX2, and PAX8 can assist with this differentiation [195]. Many previously diagnosed MuOC were in fact ovarian metastases which explains the decrease in the reported incidence over time. MuOC associated with pseudomyxoma peritonei have been shown to arise from the appendix [196]. MuOC are categorised by their growth pattern which have prognostic implications. The expansile subtype has lower metastatic potential whereas the infiltrative subtype is more aggressive and associated with a poorer prognosis with higher rates of lymph node involvement in early stage disease [197].

Unlike HGSOC, MuOC do not contain *BRCA* mutations or defects in HRR genes and have distinct gene expression profiles [197]. *KRAS* mutations occur in up to 50% of tumours [198, 199], with *c-MYC* amplifications in up to 65% of tumours [197]. HER2 gene amplification has been shown to occur in 18% of MuOC with early phase evidence supporting the use of the monoclonal anti-HER2 antibody, trastuzumab, in these tumours [200, 201]. HER2 amplification has been found to be mutually exclusive to *KRAS*. Other alterations include *TP53* mutations (50-75%), *CDKN2A/B deletions* (25%), *PI3KCA* mutations (13%), with low mutational frequencies (up to 5%) of *PTEN, BRAF, FGFR, KIT* and *STK11* [197]. 15% to 20% of MuOC harbour MSI, a finding which may make these tumours attractive candidates for immune check point inhibition [197].

The vast majority of MuOC present with early stage disease [202]. An analysis of seven phase III randomised GCIG trials found that advanced stage MuOC had a worse survival compared to serous tumours of 14.6 months versus 40.6 months, respectively [203]. MuOC also exhibit lower platinum sensitivity with response rates of between 26% and 60% [204-206] when compared to HGSOC. The mEOC trial/GOG241 compared adjuvant capecitabine and oxaliplatin versus conventional carboplatin/paclitaxel chemotherapy in stage II-IV or recurrent MuOC. This trial unfortunately closed prematurely due to poor accrual [207]. Following central pathology review, only half the patients were found to have true MuOC with the remaining tumours diagnosed as metastases to the ovary, borderline mucinous tumours and other EOC histological subtypes. Progress in the management of this rare EOC subtype has thus been hindered by its rarity, challenges in pathological diagnosis and need for a collaborative approach in carrying out clinical trials.

1.5 Endometrioid ovarian carcinoma

EnOC make up 10% of all EOC. Compared to the majority of histological subtypes of EOC, EnOC has been generally under-investigated. A comprehensive overview of the clinical, pathological and molecular characteristics of this subtype is presented in the following sections.

1.5.1 Histogenesis

Like CCOC, endometrioisis is a pre-cursor of EnOC. It is a benign condition defined by the presence of extra-uterine endometrial tissue that occurs via retrograde menstruation, and shares some characteristics of malignancy such as loss of heterozygosity and monoclonality [208].

20% to 40% of EnOC are associated with endometrioisis, either contiguous with or in relation to anatomical sites such as the contralateral ovary or pelvic peritoneum [209]. Most low grade EnOC arise from within endometriotic lesions or cysts and can occasionally co-occur with CCOC as mixed tumours in support of their shared pre-cursor lesion [174]. Malignant transformation can occur via the development of atypical endometriosis. Adenofibromas and borderline endometrioid tumours have also been found to co-exist with low grade EnOC suggesting an adenoma-carcinoma progression model [210]. Mutations in the tumour suppressor gene, *PTEN*, and *ARID1A*, in both EnOC (discussed in section 1.5.8) and adjacent endometriotic lesions also support this malignant genetic transition spectrum [7, 211, 212]. In-vivo studies have also found deletion of *PTEN* or activation of *KRAS to* induce endometriotic lesions, whereas a combination of these two mutations resulted in the development of metastatic EnOC in these mouse models [213]. Studies comparing the gene expression profiles of different histological subtypes of EOC with different gynaecological epithelia found that EnOC correlated with changes in normal endometrium, whereas serous tumours correlated with those in normal fallopian tube, further supporting the endometriotic origins of EnOC [214].

There are however studies which postulate that not all EnOC are directly related to endometrioisis and that endometrioisis-independent EnOC may differ in terms of their clinico-pathological characteristics [215-219]. Stewart et al found *KRAS* mutations to be significantly greater in endometrioisis-associated low grade EnOC compared to those with no endometrioisis [220]. Catasus et al found a higher frequency of mutations in *PTEN*, *CTNNB1* and MSI in endometrioisis associated EnOC compared to those with no endometrioisis [219]. Banz et al and Zhang et al found that endometrioisis-associated EnOC have a distinct molecular gene signature compared to EnOC without endometrioisis [216, 217]. Zhang et al also found a trend for higher stage disease in those without endometriosis [216]. Collectively, these studies suggest that EnOC may not be one disease, and that subtypes of EnOC may develop via alternative pathways.

1.5.2 Grading

The grading of EnOC is performed according to the International Federation of Obstetrics and Gynecology (FIGO) grading system for endometrial endometrioid carcinomas (EnEC) as they both share morphological similarities [221]. This is a three-tier grading system based on the percentage of non-squamous solid component present (<5% grade 1, 6%-50% grade 2 and >50% grade 3). The presence of severe nuclear atypia increases the architectural grading by 1 [222]. It is generally accepted that grade 1 and grade 2 EnOC can be grouped as low grade and grade 3 EnOC as high grade [223].

However, the prognostic value of the FIGO grading system in EnOC has been questioned by several retrospective studies. Parra-Herran et al compared FIGO grading to the Silverberg grading system which is based on architecture and nuclear atypia, mitotic activity in two high-power fields [221]. 72 patients with EnOC defined by the WHO 2014 criteria were reviewed and independently graded according to the Silverberg and FIGO grading systems. The survival outcomes of Silverberg grade 1 and 2 EnOC clustered together and were superior to Silverberg grade 3 EnOC even after five years. This is in contrast to FIGO grade 2 EnOC which had similar outcomes to FIGO grade 1 EnOC for the first five years and declined after five years approaching that of FIGO grade 3 tumours. Only the Silverberg grading system retained statistical significance for survival in a multivariate analysis when compared to FIGO grading although lost significance when accounting for stage. There was no association between the type of grading and survival in Stage I and II disease although the authors argue that the lack of association may have been limited by cohort-size and the lower number of events. The authors conclude that the Silverberg grading system is a better predictor for survival then the FIGO grading system and may favour a two tier grading classification in EnOC. This contrasts with the study by Assem et al which assessed clinical outcomes of 179 EnOC graded by the FIGO system following contemporary pathology review [224]. No differences in clinical pathological variables or survival were found between grade 2 and 3 EnOC and the authors argue that grade 2 and 3 EnOC should be grouped together instead. Together, these studies indicate that there is likely inter-observer variation in the FIGO grading system of EnOC, particular in determining grade 2. This is in keeping with similar observations made in the FIGO grading system used in EnEC [223]. For example, in the PORTEC clinical trial which compared surgery and post-operative radiotherapy to surgery alone in stage I endometrial carcinomas, up to 50% of grade 2 carcinomas were reassigned grade 1 following central pathology review [225].

1.5.3 Pathology

Most EnOC are usually well differentiated and are of low grade (grade 1 and 2). As such, they are easily distinguishable from other subtypes of EOCs based on histo-pathologic features [226]. Low grade EnOC closely resemble EnEC at both the histological and molecular level [227, 228] (discussed in section 1.5.8). Morphological features of EnOC include back to back glandular architecture with stromal exclusion and villo-glandular patterns with up to 50% of cases exhibiting squamous differentiation in the form of morules. Confirmatory endometrioid features include the presence of squamous metaplasia, background endometriosis or borderline adenofibroma, and borderline endometrioid features [226]. Other rarer morphological features include focal or diffuse sex-cord like formation, spindle cell differentiation, secretory and oxyphillic variants [129]. More than 70% of EnOC are positive for ER and PR with the classical IHC profile of EnOC being negative for WT1 staining and p53 wild-type expression (variable intensity of nuclear p53 staining). High grade EnOC are increasingly rare, and are challenging to diagnose as they share morphological overlap with HGSOC [229]. The use of WT1 is a useful discriminator (discussed in section 1.5.4) [230].

Overall, 15-20% of EnOC are associated with synchronous EnEC [129, 231]. These synchronous EnEC are usually low grade and organ confined and are normally treated as independent primary tumours. The Young and Scully criteria are used to identify both tumours as separate primaries: i) both tumours must be histologically distinct; ii) no evidence of deep myometrial invasion of the endometrial tumours; iii) no evidence of lymphovascular space invasion of the endometrial tumour; iv) presence of atypical endometrial hyperplasia; v) no evidence of other endometrial metastases; vi) unilateral ovarian tumour; vii) parenchymal confinement of the ovarian tumour with no other evidence of metastatic spread; viii) presence of ovarian endometriosis; vi) different DNA ploidy if evidence of tumour aneuploidy; and vii) different molecular genetic or karyotype of both tumours [232]. In clinical practice, however, it can still be challenging to differentiate between two independent primaries or metastatic disease either from the ovary or uterus. Notably, patients with synchronous EnEC have been shown to have a similar prognosis when compared to those without concurrent endometrial carcinomas [231, 233-235]. Interestingly, recent studies have shown that EnOC and synchronous EnEC demonstrate a clonal relationship following genomic characterisation [236, 237] suggesting an origin from a single tumour cell.

1.5.4 Diagnostic challenges in Endometrioid Ovarian Carcinomas

The biggest diagnostic challenge in EnOC is differentiating high grade EnOC from HGSOC. Other pitfalls include differentiating de-differentiated carcinomas (low grade EnOC with an undifferentiated carcinoma (UC) component) from grade 2 or high grade EnOC, as well as the exclusion of colorectal metastases with pseudo-endometrioid appearances. Rarely, EnOC can also mimic serous borderline tumours [238] and MuOC [239].The first three major areas are discussed below.

1.5.4.1 Role of WT1 in differentiating high grade EnOC versus HGSOC

High grade EnOC can be challenging to distinguish from HGSOC on the basis of morphology alone [224, 226, 229]. HGSOC is morphologically diverse and can include glandular differentiation with a papillary architecture. Solid variants of HGSOC, with minimal glandular or papillary differentiation, can mimic undifferentiated carcinomas, as well as display morphological overlap with high grade EnOC[240]. In particular, a proportion of HGSOC demonstrate solid, pseudo-endometrioid and/or transitional-cell-like growth patterns (SET pattern), which has been shown to be associated with *BRCA1* mutations [241].

Through the refinement of ovarian cancer diagnostic criteria [242] and increased use of IHC, several studies have shown that many high grade EnOC are in fact HGSOC [226, 243]. The use of WT1 IHC [230, 244, 245], is a critical tool in differentiating high grade EnOC (WT1 negative) from HGSOC (WT1 positive), and has helped reduce inter-observer variation [129, 224, 229, 243].

These findings are further supported by both gene expression profiling studies performed in EnOC diagnosed without the use of IHC [243, 246-248]. In Wu et al, 72 historically diagnosed EnOC, of which 29% were grade 3, underwent mutational analysis and 37 underwent gene expression profiling [247]. A subgroup of EnOC, which tended to be of higher grade with *TP53* mutations, had similar gene expression profiles to serous carcinomas. In Winterhoff et al, 276 HGSOC, CCOC and high grade EnOC were selected for gene expression profiling using Agilent microarrays [248]. Notably, 39% of the high grade EnOC cohort in this study were grade 2. Transcriptional profiling using TCGA signatures of HGSOC found that high grade EnOC of advanced stage clustered together with HGSOC, whereas early stage high grade EnOC and CCOC formed a distinct cluster of their own. Schwartz et al found that the gene expression profiles of advanced stage high grade EnOC clustered together with serous carcinomas, whereas early stage low grade EnOC clustered separately [246]. Similar findings were reported in Tothill et al [249].

The WT1 gene is a transcription factor involved in the development of genitourinary organs which acts as both an oncogene and tumour suppressor. It has been shown to be differentially expressed amongst gynaecological epithelia [250]. It is positive in the fallopian tube and ovarian surface epithelia but negative in the endometrium and cervix. As discussed in section 1.4.1 and 1.5.1, both HGSOC and EnOC differ in their gynaecological tissue of origin. As discussed in section 1.4.1, HGSOC have tubal origins whereas most EnOC arise from endometrioisis (ectopic endometrial tissue). As such, WT1 expression is used to demonstrate cell lineage and is differentially expressed in the different histological subtypes of EOC [244, 251]. 97% of HGSOC have been shown to be WT1 positive. An IHC profile of

WT1 positivity and p53 aberrant expression is highly specific for HGSOC (91.7%) [229, 251], whereas this combination is only observed in 1% of EnOC. Previously, 70% of EnOC were found to be WT1 negative [245, 252]. However, large studies performed of contemporary subtyping of EOC now demonstrate that 90-96% of EnOC are indeed WT1 negative [229, 253].

This is further supported by the study by Madore et al [243]. EnOC which expressed the WT1 gene clustered together with serous carcinomas, whereas EnOC which did not express the WT1 gene clustered separately. It also found that high grade EnOC which were WT1 positive p53 aberrant expression on IHC were negative for β-catenin expression, supporting these to be HGSOC that had been misclassified [243]. However, this study also identified two WT1 negative EnOC of high grade and high stage with concurrent aberrant p53 and β-catenin IHC staining. One of these tumours were immortalised in cell culture and gave rise to an aggressive well-characterised EnOC cell line, TOV112D. This study concluded that an IHC profile of WT1 negative p53 aberrant expression may identify a rare high grade variant of EnOC [243].

To date, studies which have applied contemporary ovarian sub-typing criteria have found that high grade EnOC is increasingly rare with reported frequencies of between 5-18.7% of all EnOC [224, 226, 254, 255].

1.5.4.2 De-differentiated carcinomas

De-differentiated carcinomas of the ovary contain a mix of low grade EnOC and UC components. These tumours are recognised by the WHO 2014 [256] as a separate entity and was first described by Silva et al in 2006 [257]. Microscopically, they appear as medium to large sized monotonous cells that lack glandular papillary, squamous or neuroendocrine differentiation. Most studies performed to date have largely comprised of de-differentiated endometrial carcinomas with only a few including those of ovarian origin reflecting its extreme rarity.

Recent mutational analysis of de-differentiated carcinomas of both endometrial and ovarian origin have demonstrated a clonal relationship between both the low grade endometrioid and UC components [258]. In Kuhn et al, the same somatic mutations (*PIK3CA, CTNNB1, TP53, FBXW7* and *PPP2R1A)* were found in both components in all cases [258]. In 42% of cases (n=5), additional exclusive somatic mutations were detected in UC but not found in the matched low grade endometrioid component. Subsequently, Karnezis et al found inactivating mutations of the *SMARCA4* and *SMARCB1* genes, which encode for core subunits of the switch/sucrose non-fermenting (SWI/SNF) chromatic remodelling complex, in half of the UC component, but with none in the matched low grade endometrioid component [259]. In a follow on study by Coatham et al, co-inactivation of *ARID1A* and *ARID1B* genes were identified as the other alternate mechanisms of de-differentiation [260]. The same mechanisms, including loss of BRG1 (encoded by *SMARCA4*), were also identified in the 3 ovarian de-differentiated carcinomas included in this study. Collectively, these studies support the hypothesis that the UC component develops as a result of tumour progression from the existing low grade endometrioid carcinoma, and that inactivation genes encoding the SWI/SNF complex subunits are a major mechanism of de-differentiation.

Clinically, de-differentiated ovarian and endometrial carcinomas are diagnostically challenging and can be mis-diagnosed as grade 2 or grade 3 endometrioid carcinomas. They are important to accurately diagnosis as they display aggressive clinical behaviour associated with poorer survival when compared to grade 3 endometrioid carcinomas [261, 262]. Furthermore, there is a reported association between dedifferentiated carcinomas of gynaecological origin and MMR deficiency, with loss of MMR proteins on IHC in over 50% of de-differentiated endometrial carcinomas [257, 261, 263, 264]. As such, it is likely that accurate diagnosis of de-differentiated ovarian carcinomas may also have implications in screening for Lynch syndrome.

1.5.4.3 Pseudo-endometrioid colorectal metastases

Ovarian metastases from colorectal carcinomas are a known mimic of primary ovarian carcinomas and can occur in 10-33% of cases [265, 266]. In particular, they can display pseudo-endometrioid appearances [265]. The coordinate use of CK7 and CK20 can help distinguish non-mucinous ovarian carcinomas (CK7 positive CK20 negative) from colorectal metastases (CK7 negative, CK20 positive) and have been validated by a large number of studies [267, 268].

1.5.5 Hormone receptor expression

Most EnOC express ER and PR [81].The relative risks of developing EnOC following oestrogen replacement therapy have been found to be one of the highest amongst the histological subtypes of EOC [69, 269]. In keeping with this, the risk reducing effect of the oral contraceptive pill has been found to be one of the greatest for EnOC [70]. Furthermore, Kuhnel et al found the highest activity of aromatase in EnOC compared to serous and mucinous ovarian carcinomas, suggesting that in situ oestrogen production may be an important growth factor in EnOC, and as such may benefit from the use of aromatase inhibitors [270]. Like EnEC, these findings demonstrate that EnOC is an oestrogen dependent disease.

The Ovarian Tumour Tissue Analysis Consortium Study was the largest study of over 3000 patients evaluating ER and PR expression between the histological sub-types [81]. As previously discussed in section 1.3.1, three categories were used to define hormone receptor expressions according to the proportion of nuclear staining (strong (≥50%), weak (1%-50%) and negative (0%)). 76.6% of EnOC (n=484) expressed ER with 60.2% displaying strong expression and 16.4% displaying weak expression. Similarly, 67.4% of EnOC expressed PR with 44.4% displaying strong expression and 23.0% weak expression. The majority of EnOC (81.6%) had co-expression of ER and PR. This study found that any ER and PR expression (defined as >1% nuclear staining) was associated with an improved disease-specific survival (DSS) in EnOC. It also found that high grade EnOC were less likely to be ER or PR positive when compared to their lower grade counterparts. Co-expression of ER and PR were also evaluated in this study. The independent risk reduction on DSS of ER (positive or negative) and/or PR (positive or negative) expression was similar for all groups.

EnOC in the Ovarian Tumour Tissue Analysis Consortium Study did not undergo contemporary pathology review [81]. In order to address the impact this may have had on the study results, WT1 IHC expression was examined in a subset of HGSOC. It found no differences in the frequencies of WT1 expression in strong, weak and negative PR (P=0.61), thus concluding that the misclassification of EnOC as HGSOC was not more likely for PR positive tumours.

Rambau et al performed independent validation of the Sieh et al study specifically in EnOC [255]. Here, contemporary pathology review utilising an eight marker IHC algorithm identified 182 EnOC of which 19% were grade 3. Using the same scoring methodology as Sieh et al, frequencies of ER and PR expression were 87.3% and 86.7%, respectively. Co-expression occurred in 83.0%. There were no significant differences in DSS between focal (1-50% nuclear staining) and diffuse (>50% nuclear staining) staining and a binary approach for survival analyses was thus adopted. Any ER or PR expression was associated with superior DSS in EnOC independent of age, stage, grade, treating centre and residual disease, in keeping with those found by Sieh et al. However notably, the effect on DSS was diminished when restricted to stage I and II disease. Only borderline significance was observed for ER positive (P=0.0452) and ER and PR co-expressed EnOC (P=0.0452), whilst no statistically significant differences were observed for PR (P=0.0932).

Very few studies have been performed evaluating the role of AR in EnOC as many of them grouped non-serous tumours together. The rate of AR expression in small cohorts vary between 20%-58% (Table 5). Like studies of ER and PR, variable thresholds of defining receptor positivity have been used.

Furthermore, no study has assessed the prognostic role of AR specifically in EnOC due to the low numbers of EnOC present in each study. The only study that has come close to doing so was performed by Jonsson et al [83]. Here, 118 tumours of both serous (73.7%) and endometrioid histology (26.3%) were analysed and scored for ER, PR and AR expression. An IHC threshold of ≥ 10% was considered positive expression. In the whole cohort, both PR and AR expression, but not ER, were independently associated with improved prognosis. Co-expression of PR and AR was also found to provide a greater differential effect on survival compared to tumours with discordant or absent PR and AR expression. A trend was seen when the latter analysis was restricted to EnOC only (P=0.073). However, residual disease was not recorded in this study thus representing a major limitation to this study. The independent prognostic role of AR has thus not been established in EnOC.

Table 5: Studies of AR expression in EnOC.

Study	IHC threshold	N of EnOC included	AR expression in EnOC
Lee et al 2005[a] [87]	>10%	29	58%
De Toledo et al 2014[a][94]	Allred score≥4	14	not recorded
Van Kruchten et al 2015 [a][95]	≥ 10%	21	not recorded
Nodin et al 2010[a][90]	>10%	35	20%
Jonsson et al 2015[a][83]	≥ 10%	31	25%
Gomora et al 2018[a][105]	Histoscore>30	29	Over 40%[b]

Legend: IHC=immunohistochemistry; N=number; AR=androgen receptor.
[a] performed in mixed population of epithelial ovarian carcinomas.
[b]exact number not recorded.

1.5.6 Clinical characteristics

EnOC are more commonly diagnosed in younger women compared to those with HGSOC [271, 272]. It usually presents as a large pelvic mass comprised of low grade carcinoma with the majority presenting as early stage disease [226]. Most EnOC are unilateral though 10-20% can develop as bilateral lesions [209, 210, 272]. As previously discussed, up to 40% of EnOC are associated with endometriosis [129, 209] with 15-20% associated with synchronous EnEC [273].

In general, EnOC is regarded as having a better prognosis then HGSOC [226, 229, 272, 274, 275]. It is however less certain whether this is solely due to earlier stage of presentation as most studies did not perform stage for stage comparisons [274, 276-278]. Furthermore, older studies of EnOC did not undergo contemporary pathology review [272, 279]. As discussed in section 1.5.4.1, many historically diagnosed high grade EnOC are in fact HGSOC and are likely to have confounded the clinical outcomes reported in these studies.

A meta-analyses initiated by the GCIG investigated the prognostic relevance of histological subtype [203]. Here 8704 patients with stage III/IV EOC from seven randomised clinical trials (three of which underwent central pathological review) were included, of which 646 (7.4%) were EnOC. Patients with EnOC were more likely to have no gross residual disease compared to serous histology, independent of age and study. The median OS was 50.9 months for EnOC compared to 40.8 months in serous histologies, although this was not statistically significant [203].

Storey et al performed evaluating a stage for stage comparison of serous carcinomas and 270 EnOC diagnosed between 1984 and 2004 [272]. EnOC had superior overall median PFS (24 months versus 13 months; $p<0.0001$), and overall median OS (48 months versus 22 months, $p<.0001$), when compared to serous carcinomas. This held true for stage II and III disease, however there were no differences observed for stage I and IV disease. Histology, stage and residual disease were independently associated with survival following platinum based chemotherapy. However, this dataset did not undergo contemporary pathology review with 56% of EnOC in this study comprised of grade 3 tumours. It is thus plausible that a significant proportion of these tumours were misdiagnosed HGSOC [280]. Furthermore, the distinction between HGSOC and LGSOC was also not made in this study with nearly a third of serous carcinomas comprised of grade 1(7%) and 2(22%) serous carcinomas. Together, these factors are likely to have confounded the results of this study. In another similar example, Bouchard Fortier et al compared 98 EnOC to 435 HGSOC [279]. No pathology review was performed. Five year OS was significantly higher at 80.6% in EnOC versus 35.0% in HGSOC although this lost significance on a multivariate analysis including stage.

A few studies have compared contemporary defined EnOC to HGSOC. In Kobel et al, 185 EnOC were retrospectively diagnosed utilising WT1 IHC with 7.6% of patients diagnosed as G3 [281]. Ten year DSS was 96% for stage IA/IB EnOC compared to 68% for HGSOC, and 81% for stage IC-II EnOC compared to 57% for stage IC-II HGSOC. It is however worth noting that in this study, tumours were considered stage II if sharp dissection was required even in the absence of pathologically proven extra ovarian spread which is not standard practice worldwide. These patients, who would otherwise be

considered stage I, therefore received adjuvant therapy. Furthermore, over half of the study cohort also underwent abdominal pelvic radiation in addition to adjuvant chemotherapy. These practices, which are not considered standard of care worldwide, may pose some limitations on the interpretation of clinical outcomes reported in both studies. Nonetheless, this contributes to the growing body of data supporting the belief that EnOC demonstrate biologically superior outcomes compared to HGSOC.

Lim et al performed contemporary pathology review on 109 historically diagnosed EnOC as per WHO 2014 criteria [226]. Of these, 70% were classified as true EnOC. Only 31 tumours, excluding high grade EnOC, underwent WT1, p53 and p16 IHC on tissue microarrays. The rate of high grade EnOC was 2.6% for Silverberg grading and 5.3% for FIGO grading. Compared to HGSOC also diagnosed through the same pathology review process, EnOC were found to present at an earlier age, stage, with low grade unilateral disease. They were also more likely to have no evidence of disease at last follow up. However, no stage for stage comparison was performed in this study.

Some studies have tried to specifically compare contemporary defined high grade EnOC to HGSOC. In the study by Assem et al, 30 high grade EnOC diagnosed as per WHO 2014 utilising 10 IHC markers including WT1 and p53 IHC on TMAs were compared to HGSOC [224]. De-differentiated carcinomas were excluded. This study found the survival of high grade EnOC to be superior to that of HGSOC on univariate analysis, but not on multivariate analysis. It also found that grade 2 and grade 3 EnOC had similar outcomes and postulated that these 2 groups could be combined, in contrast to the convention of grouping grade 1 and 2 EnOC together. In Soyama et al, WHO 2014 defined grade 3 EnOC comprising 13.8% (n=9) of EnOC were compared to HGSOC [282]. Notably, IHC performed at first diagnosis was used only if there were discrepancies between the two observers, with no specific details regarding this found in this paper. No differences in PFS or OS were found between the 2 groups. In summary, the published evidence to date suggests that the clinical behaviour of high grade EnOC is similar to that of HGSOC.

1.5.7 Clinical Management

As discussed in section 1.2, the principles of management of EOC largely reflect that of HGSOC due to its prevalence in prospective randomised trials. In general, the approach to the management of EnOC is similar to that of HGSOC. No established EnOC-specific management exist as no clinical trials have been performed specifically in this histological subtype. In particular, most modern day clinical trials often combine high grade EnOC with HGSOC. In this chapter, the treatment settings in which the management of EnOC may differ to that of HGSOC are discussed.

1.5.7.1 Surgery in early stage EnOC

In stage I disease, EnOC is one of the histological subtypes in which fertility sparing surgery can be considered. The European Society of Medical Oncology guidelines recommend that following appropriate counselling, this surgical procedure can be performed in younger pre-menopausal patients with stage IA or stage IC grade 1 and 2 EnOC, provided complete surgical staging, including lymphadenectomy, is performed to exclude more advanced disease [2].

As discussed in section 1.2.1, the role of lymphadenectomy in early stage disease is uncertain as no OS benefit derived from a prospective randomised trial has been demonstrated [17]. Its practice is controversial and variable worldwide. Several large retrospective studies have evaluated the prevalence of lymph node metastases according to histological subtype, and the impact of lymph node sampling or lymphadenectomy on survival. Heitz et al evaluated the role of lymphadanectomy in EOC with data extracted from a prospectively maintained database [283]. Of the 27 grade 1 EnOC in this cohort, no lymph node metastases were observed independent of stage. In contrast, the rate was 27.6% in grade 2 and 3 EnOC which was stage dependent, compared to 67.1% in HGSOC. Although there was a survival advantage of lymphadenectomy in non-serous tumours, there were no differences in survival specifically in EnOC. In Minig et al, the frequency of lymph node metastases following lymphadenectomy was also found to be similarly low at 1.5% of 68 historically diagnosed apparent stage I low grade EnOC [284]. Lymphadenectomy was not associated with survival. Similarly, in Melamed et al, the rate of lymph node metastases was 1.6% of 1120 historically diagnosed apparent stage I EnOC, compared to 34.5% in serous carcinomas [285]. In the study performed by Kobel at al which evaluated clinical outcomes of contemporary reviewed early stage EnOC, patients did not routinely undergo lymph node dissection. DSS was 96% in apparent stage IA and IB EnOC with no adjuvant therapy suggesting that radical staging surgery may be safely avoided in these patients.

Conversely, other retrospective studies have concluded that lymphadenectomy remains an important determinant of survival even in EnOC. Nasioudis et al investigated the frequency of lymph node metastases and prognostic role of lymphadanectomy in apparent stage I EnOC and MuOC [286]. 17.8% of this historically diagnosed EnOC cohort were grade 3.Only 2.1% of 3354 EnOC had lymph node metastases. On multivariate analyses, lymph node sampling or lymphadanectomy was associated with DSS only in EnOC. In a large national Dutch retrospective study evaluating the role of lymphadenectomy in early stage EOC (stage I-IIA and IIIA1) of which 354 were EnOC, OS was superior in patients who underwent lymphadenectomy compared to those who did not, even after correcting for histology, grade and stage [287]. The proportion of grade 3 EnOC was however not reported in this

study. In a Chinese study of 78 stage I historically diagnosed EnOC of which 21.8% were grade 3, grade 3 disease and those who underwent lymphadenectomy were independent factors of prognosis [288]. In another large American study abstract of 3617 patients with historically diagnosed stage I EnOC, the removal of more than 10 lymph nodes was independently associated with improved DSS after adjusting for age, sub-stage, tumour grade and chemotherapy (P=0.01) [289]. No information was available on the proportion of patients with grade 3 disease or rates of lymph node metastases. In all four studies, no contemporary pathology review was conducted and there is thus some uncertainty regarding the proportion of misdiagnosed HGSOC included in these cohorts.

Collectively, the data to date suggest that the rates of lymph nodes metastases in EnOC are low particularly in low grade tumours. The question as to whether a survival benefit exists for lymph node sampling or dissection specifically in EnOC is uncertain due to conflicting data. In particular, the possible inclusion of misdiagnosed HGSOC in these historically diagnosed cohorts may have influenced these results.

1.5.7.2 Adjuvant chemotherapy in early stage EnOC

Both the ICON1 and ACTION trials of adjuvant chemotherapy in EOC did not distinguish between each histological subtype and thus the survival benefit of adjuvant platinum-based chemotherapy specifically in EnOC is less well defined [27]. Only 24.1% (n=120) of patients in the ICON and ACTION trials were EnOC, Retrospective subgroup analysis of these two trials did not demonstrate a differential effect of chemotherapy for histological subtype. In a ten year update of the ACTION trial, the observed survival differences between the chemotherapy and observation arm were independent of histological sub-type [290].

The majority of early stage EnOC are associated with good prognosis, in particular, five year DSS of stage I EnOC is over 90%. As such, retrospective studies have been performed evaluating the value of adjuvant chemotherapy in early stage disease [274, 281, 291]. In addition, attempts have been made to identify biomarkers which can define a subgroup of patients that can be spared adjuvant chemotherapy.

The largest retrospective analysis performed by Nasioudis et al investigated the role of adjuvant chemotherapy in 4538 patients from the United States National Cancer Database with historically diagnosed stage 1 EnOC [292]. The use of lymphadenectomy was used as a surrogate marker of adequate surgical staging. Here, a statistically significant OS advantage was observed in patients with grade 2 tumours with no lymph node dissection or who had limited lymphadenectomy who received adjuvant chemotherapy versus those who did not. A numerical but non statistical OS advantage was also observed in those with grade 3 disease with no lymphadenectomy who received adjuvant chemotherapy. The authors conclude that there is a survival benefit for patients with inadequately staged grade 2 EnOC and possibly those with grade 3 EnOC, however acknowledge the limitations of this study which include the lack of contemporary pathology review, as well as the use of OS rather than DSS as a survival endpoint.

In Oseledchyk et al, the survival benefit of adjuvant chemotherapy was evaluated in 3552 historically diagnosed stage I EnOC diagnosed between 2000 and 2013 as part of the SEER database [291]. In this study, 75% of patients had at least one lymph node removed, and 17.6% were G3 EnOC. No differences in overall five year OS were found between patients who received adjuvant chemotherapy and those who did not (90% versus 89%, respectively; P=0.807). Only patients with stage IC G3 EnOC demonstrated a survival benefit from chemotherapy (five year OS of 81% versus 62%, P=0.003), whereas those with stage IA/IB any grade, and IC grade 1 or 2 EnOC did not derive benefit [291].

Rambau et al evaluated the prognostic and predictive role of hormone receptor expression in a group of contemporary defined EnOC utilising IHC [255]. Five year DSS was excellent at 97.8% in stage IA/IB disease. In stage IC/II disease, no differences in five year DSS were found for tumours which expressed ER, PR or ER/PR co-expression versus those which did not. The authors conclude that for these patients, hormone receptor expression was of limited value in identifying those that could be spared adjuvant chemotherapy. For example, five year DSS was 89.0% and 89.9% in ER and PR positive tumours, respectively, which was lower than the 95% threshold defined in the study at which patients could avoid chemotherapy. Akin to the study by Oseledchyk et al, no differences in survival were observed in stage IC/II disease between those who received adjuvant chemotherapy versus those who did not (P=0.32), raising the hypothesis as to whether adjuvant chemotherapy could be omitted in these patients. Similarly, no differences were observed between these groups in ER positive (P=0.76) or negative disease (P=0.19).

Kumar et al evaluated clinical outcomes in 172 early stage EnOC [274]. No contemporary review was performed in this study and 12% were G3 EnOC. Five year DFS rates were 95% for stage IA-C (IC by tumour rupture only), 84% for stage IC (positive cytology and/or surface involvement), and 74% for stage II disease, respectively [274].The authors found 2 poor prognostic groups, patients older than 55 years with stage IC (positive cytology and/or surface involvement) and any stage II patient. This study concluded that patients with stage IA/B and stage IC EnOC defined by tumour rupture alone could avoid adjuvant treatment. Like the study by Kobel et al [281], tumours requiring sharp dissection were considered stage II even with pathologically negative extra-ovarian spread, and a proportion of patients also underwent adjuvant radiotherapy in addition to chemotherapy. A trend in favour of DFS benefit for radiotherapy in early stage EnOC was observed, although the confidence intervals crossed 1 (RR 1.77 (95% CI 0.74-4.24).

Other studies have also evaluated the role of other molecular markers to identify patient cohorts who could be spared chemotherapy. Wang et al investigated the role of β-catenin and CDX2 IHC staining in both a discovery (n=183) and validation cohort (n=174) [293]. Contemporary pathology review was performed using IHC. Both markers were individually associated with DSS on univariable analysis. In a multivariable analysis that only accounted for stage, double positivity of both markers identified patients with stage IC/II EnOC that could be spared chemotherapy in the discovery cohort (ten year DSS greater than 95%), however this lost significance in the validation cohort. Notably, this study did not have surgical procedures or residual disease recorded and the authors acknowledge the need to evaluate

other prognostic markers of EnOC as a multi-marker panel in order to establish the utility of both beta-catenin and CDX2 as prognostic biomarkers.

Soovares et al evaluated the role of L1CAM expression in 249 EnOC which underwent pathology review (though no details were outlined in the paper) [275]. On univariable analysis, higher levels of L1CAM expression were found in EnOC of advanced stage, high grade with residual disease following surgical cytoreduction compared to those of early stage, low grade disease with no residual disease. Although positive L1CAM expression was associated with poorer survival in EnOC upon univariable analysis, this lost significance upon multivariable analysis, limiting the clinical utility of this biomarker in identifying patient cohorts of EnOC which could be spared adjuvant chemotherapy.

1.5.7.3 Management of advanced stage EnOC

In general, the management of advanced stage and relapsed EnOC mirrors that of HGSOC. Following aggressive cytoreductive surgery and platinum based chemotherapy, maintenance PARP inhibition can be employed for germline *BRCA* mutated high grade EnOC as they were included in the original PARP inhibitor trials [294]. As discussed in section 1.4.5, EnOC is recognised to be a hormone sensitive cancer in particular due to high co-expression of both ER and PR. Endocrine therapy in EOC is unlicensed however phase II clinical trials and retrospective studies demonstrate that the degree of ER expression predicts for endocrine sensitivity [112, 113, 295]. The published evidence of use of endocrine therapy in EnOC is largely confined to case reports and sub-group analyses of retrospective studies. In Pan et al, two patients diagnosed with stage IIIC EnOC were treated with the aromatase inhibitor, letrozole, as maintenance treatment [296]. The first patient had treatment initiated following first line surgery and chemotherapy following the discovery of residual disease during a second-look laparoscopy. In the second patient, letrozole was initiated following secondary cytoreduction and chemotherapy following a third relapse after an 11 month remission. Both patients remained disease free for 30 months thereafter. In George et al, retrospective analysis of endocrine sensitivity in EOC was performed [222]. Of the five high grade EnOC in this cohort, three had a partial response, and two demonstrated stable disease. Endocrine therapy, a low cost and well tolerated drug, could therefore be conceivably used for recurrent EnOC, or as maintenance therapy in high risk patients not fit for chemotherapy. In EnEC, in which EnOC is histologically and molecularly similar to, the use of endocrine therapy is commonly administrated for recurrent low grade disease despite the lack of prospective trial evidence [297], further supporting this approach in EnOC.

1.5.7.4 Platinum sensitivity of EnOC

Finally, as discussed in sections 1.3, each histological subtype of EOC have differential platinum sensitivity, although this has been under-investigated specifically in EnOC. To date, only three studies have reported on platinum sensitivity specifically in EnOC. Storey et al clinically characterised 270 historically diagnosed EnOC of which more than half were grade 3. According to WHO/UICC criteria, it found EnOC to display similarly high objective response (59%) and CA125 response rates (66%) to platinum based chemotherapy when compared to serous carcinomas (the majority of which were of high grade) [272]. There has only been one other study performed in 145 historically diagnosed EnOC in 1990 which reported a 72% platinum response rate in historically diagnosed EnOC, however this was not according to modern criteria [298]. In Soovares et al, 249 patients with EnOC which underwent pathology review were characterised [275]. Platinum response was evaluated after primary surgery and adjuvant chemotherapy based on gynaecological examination, vaginal ultrasonography, CA125 measurement, and/or computed tomography imaging. Overall complete response was 79%. This methodology is highly flawed given the heterogeneous assessment of response. Furthermore, they did not restrict the response analysis to those with measurable residual disease post-operatively thus casting doubt on these high response rates observed. As such, the platinum sensitivity specifically in contemporarily defined EnOC is not well understood.

1.5.8 Genetic profile of EnOC and comparisons with EnEC

Mutations in the PI3K/AKT pathway and Wnt/*β*-catenin signalling pathway are prevalent in EnOC. Commonly mutated genes include *PTEN (20%), ARID1A (30%), PIK3CA (20%), PPP2R1A* and *CTNNB1 (16-54%)* [227, 247, 254]. Mutations in *KRAS* (20%) [299], a member of the MAP kinase pathway, as well as microsatellite instability (MSI) (7-14%) [227, 300, 301], as a result of mutations in the MMR genes, are also associated with EnOC. *TP53* mutations are rare in low grade EnOC and have been reported to occur in less than 10% [228]. In studies which evaluate *TP53* mutation status by IHC, higher frequencies of up to 30% are observed when accounting for EnOC of all grades [224, 243, 254, 302].

In addition to similar morphology, EnOC and EnEC share common molecular alterations but with differing frequencies particularly in *CTNNB1*, *PTEN* and MSI in a number of studies [219, 227, 228]. Only two studies have carried out direct comparisons, each utilising different molecular detection techniques and cohorts for comparison. McConechy et al performed targeted exon capture on a panel of seven commonly mutated genes in both low grade EnOC and low grade EnEC [228]. It found low grade EnOC to have fewer *PTEN* mutations (16.6% versus 53.3%, p=0.0001) but a higher frequency of *CTNNB1* mutations (53.3% versus 27.5%, p=0.020) when compared to low grade EnEC [228]. No differences in *ARID1A*, *PIK3CA*, *KRAS*, *PPP2R1A* or *TP53* were observed. Only three high grade EnOC were identified in this study however all three had differing mutations in different genes. High grade EnEC shared similarly high frequencies of *PTEN* and low frequencies of *CTNNB1* mutations compared to low grade EnEC.

Huang et al compared EnOC (27% G3) without synchronous endometrial carcinomas to EnEC (12% G3) without concurrent ovarian involvement [227]. They showed EnOC to have lower frequencies of the MSI-high phenotype (4% versus 29%, P=0.012), loss of *PTEN* IHC expression (38% versus 74%, P=0.004) and loss of *ARID1A* IHC expression (12% versus 33%, P=0.044). The frequency of MSI in EnOC when including those with concurrent EnEC rose to 11%. There were no differences in the frequency of *KRAS* or *PIK3CA* mutations as detected by PCR between both cohorts. However, unlike McConechy et al, there were also no differences in *CTNNB1* mutations. This study also found no relationship between grade of tumour and any of the molecular aberrations. In both the EnOC alone and EnEC alone cohorts, MSI-high tumours demonstrated significantly higher *ARID1A* loss compared to MSI-low tumours. Whereas in the EnOC alone cohort, loss of *PTEN* was also significantly higher in the MSI-high group.

Both EnOC and EnEC share common pre-cursors. The development of EnEC most commonly occurs in post-menopausal women with high levels of unopposed oestrogen as a result of obesity leading to the proliferation of endometrial epithelial cells and tumorigenesis [303]. Conversely, most EnOC arise from endometriotic cysts which develop as a result of retrograde menstruation. Here, trapped menstruation blood within the confined space of a cyst leads to high iron concentrations causing oxidative stress resulting in DNA damage and an accumulation of mutations [304, 305]. This is in keeping with the studies that have shown higher levels of chromosomal abnormalities in ovarian endometriotic cysts compared to extra-ovarian endometriosis [306]. These different mechanisms of tumorigenesis and tumour microenvironment in both EnOC and EnEC may explain the differences in mutation profiles observed in both studies by Huang et al and McConechy et al.

In the upcoming sections, the molecular aberrations of interest in EnOC are discussed in detail.

1.5.8.1 *PTEN* and *PIK3CA*

Mutations in the *PTEN*-PI3K pathway occur in a wide range of malignancies. They are common in the endometrioisis-associated ovarian carcinomas, EnOC and CCOC. Phosphoinositide 3-kinases (PI3K) phosphorylates downstream signalling molecules including protein kinase B (AKT), which are pivotal to numerous cell functions including cell proliferation and migration[307]. *PIK3CA* is an oncogene which encodes for the p110alpha catalytic subunit of PI3K. Gain of function *PIK3CA* mutations result in increased PI3K activity promoting phosphatidylinositol (3, 4, 5)-triphosphate (PIP_3) mediated AKT activation thus promoting cancer cell growth. Missense mutations in exon 9 and 20 are the most common in EOC [308].

PTEN is a tumour suppressor gene on chromosome 10q23 which antagonizes the PI3K/AKT pathway through dephosphorylating PIP_3, thus inhibiting cell proliferation and survival. Inactivation of *PTEN* through loss of heterozygosity (LOH), genetic and epigenetic mechanisms, results in AKT activation initiating cancer cell growth and altered metabolism [309]. Germline mutations in *PTEN* result in the *PTEN* hamartoma tumour syndrome, including Cowden's disease. Two thirds of patients with Cowden's disease harbour mutations in exon 5, 7 and 8 with nearly half occurring in exon 5. Somatic mutations are typically frameshift mutations that can occur throughout the whole gene [310]. Both splice site mutations, as well as epigenetic silencing of the gene through methylation of the *PTEN* promoter, can also occur. LOH at 10q23.3 has been reported in up to 42.1% and 60% of EnOC [311, 312].

Both inactivating mutations *PTEN* [228, 312, 313], and activating mutations of *PIK3CA* [307], have been shown to occur in up to 20% of EnOC. LOH and *PTEN* mutations have been found in both endometrioisis, EnOC and CCOC [311], postulating this to be an early event in the development of these carcinomas.

1.5.8.2 *ARID1A*

ARID1A is a large tumour suppressor gene comprising 20 exons, which codes for the protein, BAF250a, a subunit of the SWI/SNF chromatin remodelling complex [314]. This complex modifies the expression of several genes. It interacts directly with *TP53,* and regulates the expression *of SMAD3, CDKN1A (p21), MLH1* and *PIK3IP1* through downstream transcription modification. This controls cell proliferation through the PIK3/AKT pathway.

Loss of *ARID1A* tumour suppressor function plays a pivotal role in the pathogenesis of endometriosis associated gynaecological malignancies [315]. In EOC, *ARID1A* mutations are mutually exclusive to CCOC and EnOC, with none in HGSOC [315]. The highest frequencies occur in CCOC between 46-57% [177]. Up to 30% of EnOC have been reported to contain *ARID1A* mutations and have also been found in contiguous atypical endometrioisis suggesting *ARID1A* mutations to be an early mutagenic event [315, 316].

Heterozygous truncating mutations (nonsense or frameshift (out of frame) mutations) and missense mutations [314, 317] of the *ARID1A* gene are most common whereas in-frame insertions and deletions are rarer (5%) but are also pathogenic [318]. Mutations in *ARID1A* occur across the gene with no hotspot mutation and occur as somatic but not germline mutations [177, 315]. Both heterozygous and homozygous *ARID1A* mutations correlate with loss of *ARID1A* protein expression on IHC suggesting either haplo-insufficiency or alternative mechanisms for loss of protein expression [315, 319].

The prognostic role of *ARID1A* mutational status (mutations and IHC expression) has been evaluated across different cancers in several studies. The largest meta-analysis of more than 5000 patients with gynaecological, urological and gastrointestinal cancers found tumours which were *ARID1A* deficient, defined by mutational and IHC analysis, had an increased cancer-specific relapse (HR1.93) and mortality (HR 2.55) as compared to *ARID1A* proficient tumours [320]. In EOC, the prognostic role of *ARID1A* mutational status determined by IHC has been studied the most in CCOC. Yokoyama et al found that amongst EOC, loss of *ARID1A* IHC expression was highest in CCOC and was associated with platinum resistance and worse PFS compared to tumours with intact expression [321], a finding that was also replicated in Katagiri et al [322]. Itamochi et al found that loss of *ARID1A* expression was independently associated with inferior five year survival in early stage CCOC (91% versus 74%; P=0.0225), but not in advanced stage disease [323].

In other endometrioisis-associated carcinomas, the prognostic role of *ARID1A* mutational status is less well defined. Heckl et al found loss of *ARID1A* IHC expression, together with p53 and β-catenin, to be independent variables of poor prognosis in a small cohort of historically diagnosed EnOC, CCOC, EnEC and clear cell endometrial carcinomas [324]. Mao et al correlated progressive loss of *ARID1A* expression to different stages of EnEC progression (0% complex atypical hyperplasia, 25% low grade endometrioid, and 44% high grade endometrioid)), highlighting its role in tumour progression [325]. In contrast, *ARID1A* mutations were not associated with OS in other studies of predominantly clear cell endometrial carcinomas [326-328]. Similarly, Lowery et al found no association between loss of *ARID1A*

IHC expression and survival in a cohort of 212 historically diagnosed CCOC and EnOC [329]. To date, no studies have evaluated the prognostic role of *ARID1A* mutation status solely in EnOC.

1.5.8.3 *CTNNB1*

The *CTNNB1* gene encodes for *β*-catenin which is a membranous protein which regulates cell adhesion as part of the E-Cadherin:catenin adhesion complex [330]. It is a key downstream transcriptional factor in the canonical WNT signalling pathway and is a well-established cancer signalling pathway that occurs in a range of malignancies including colorectal and ovarian carcinomas. In a normal cell, the levels of β catenin are controlled by the adenomatous polyposis coli (APC) tumour suppressor protein complex which degrades free cytosolic β catenin through the ubiquitin-proteasome pathway [330]. Upon activation of the Wnt pathway, the breakdown of β-catenin is inhibited resulting in its accumulation and translocation into the nucleus where it activates target genes such as cyclooxygenase, cyclin D and c- resulting in genomic instability [330]. Nuclear accumulation of the β-catenin protein on IHC is thus a useful surrogate for *CTNNB1* mutations and has been shown to be associated with squamous differentiation, lower grade and with endometrioid histology [293, 331, 332].

Activating mutations in the *CTNNB1* gene has been reported in 16-54% of EnOC and are usually hotspot missense mutations of exon 3 [219, 227, 228, 254, 293, 301, 332, 333]. The wide range of mutation frequencies have been attributed to various methods of detection which included IHC and different sequencing methods. The most recent study by McConechy et al 2014 performed both exon capture sequencing (n=33) and Sanger sequencing of hotspot exon 3 (n=20) of *CTNNB1* in low grade EnOC [228]. 50% of these samples had *CTNNB1* mutations all of which were missense mutations.

CTNNB1 mutational status (mutations and IHC expression) in EnOC have also been shown to be associated with good prognosis in a small number of studies [254, 293, 334, 335]. The largest study ever performed was by Wang et al 2018 who examined the prognostic role of nuclear β-catenin and CDX2 expression on IHC in 357 patients with EnOC, 183 were in the discovery set and 174 were in the validation set [293]. Contemporary pathology review was applied. In keeping with the literature, nuclear β-catenin expression was higher in grade 1 EnOC (81.2%) compared to 11.8% in grade 3 EnOC (P=0.003). It was also associated with squamous differentiation (P<0.001). No differences in expression were observed for stage. Nuclear β-catenin expression was associated with longer DSS upon univariable analysis in both the discovery and validation set. In the multivariable analysis which only accounted for stage but not residual disease, nuclear β-catenin expression together with CDX2 positivity was independently associated with DSS in the discovery cohort but not the validation cohort.

In contrast, *CTNNB1* mutations are associated with poor prognosis in EnEC. In Liu et al 2014, comparison of two transcriptomic clusters characterised by the endometrial TCGA (The Cancer Genome Atlas) was performed [336]. Both clusters were comprised of low stage, low grade disease without *TP53* mutations. Cluster II, made up of younger obese patients, demonstrated a significantly higher proportion of *CTNNB1* exon 3 activating mutations with poorer survival as compared to Cluster I (87.0% versus 17.4%; P<0.001) which had higher levels of MSI-high tumours and expression of

hormone receptors. Cluster II was found to have the lowest mutation rate amongst the four clusters of the endometrial TCGA, suggesting that the aggressive biology of this cluster was driven by *CTNNB1* mutations. Similarly in Kurnit et al, *CTNNB1* mutations, together with *TP53* mutations and age, were independently associated with worse RFS in early stage (I and II) and low grade (I and II) EnEC [337]. Furthermore, Myers et al also demonstrated an association between *CTNNB1* mutations and risk of relapse in stage IA, grade 1 EnEC which is typically regarded as having an excellent prognosis with a cure rate of above 95% [338].

1.5.8.4 *KRAS*

The RAS family of oncogenes comprise *KRAS*, HRAS and NRAS, all of which have been associated with the promotion of malignancies. The *KRAS* gene resides on chromosome 12p12 and encodes the GTPase transductor protein, *KRAS*. It is involved in the MAP-kinase signal transduction pathway and is responsible for a vast range of cellular processes. In normal cells, *KRAS* migrates between an active (GTP-bound) and inactive (GDP bound) state. Activating point mutations in *KRAS* most commonly occur in exon 1 at position 12 and 13 near the GTP binding site [339]. This results in the cells inability to hydrolyse GTP and are thus locked in a permanent active state. This causes downstream activation of the MAP-kinase pathway, driving carcinogenesis.

12% to 33% of EnOC are reported to have *KRAS* mutations [220, 227, 228, 254, 340]. Notably, studies have also found that *KRAS* mutations are mainly associated with endometrioisis [340, 341]. In Stewart et al, 29% of low grade EnOC with endometrioisis had *KRAS* mutations, compared to 3% without endometrioisis. Higher frequencies were also observed in endometrioisis identified in the ovarian tumour compared to extra-ovarian sites (32% versus 13%). In this study, the rate of *BRAF* mutations was extremely low at 1.4%, a finding similar to the low frequencies found in EnEC and unlike that of LGSOC [342]. Similarly in a follow on study also by Stewart et al performed in all grades of EnOC, the frequency of *KRAS* mutations was 25.7% in endometrioisis associated EnOC, compared to 6.3% in endometrioisis negative EnOC [340]. It is however worth noting that 12% of this cohort were WT1 positive, and nearly 10% had p53 mutant expression on IHC, thus raising the possibility that misdiagnosed HGSOC may have been included in the cohort [340]. Nonetheless, the association of *KRAS* mutations with endometrioisis is further supported by in vivo work performed by Dinulescu et al in 2005 [213]. Here, activation of *KRAS* induced both peritoneal endometrioisis and benign ovarian epithelial lesions with glandular endometrioid morphology. A further loss of *PTEN* resulted in the development of invasive EnOC. Together, these data support *KRAS* mutations to be an early event in the pathogenesis of EnOC.

1.5.8.5 Mismatch repair deficiency and microsatellite instability

The MMR system is important in maintaining genomic fidelity [343]. It recognises and repairs small base-base insertion/deletion mutations that occur frequently during DNA replication due to errors made by DNA polymerase. It also repairs mis-paired bases caused by exogenous chemicals (e.g. cigarette smoke, asbestos), environmental factors (e.g. ultraviolet light) as well as endogenous reactive oxygen and nitrogen species [344]. The MMR system therefore prevents the accumulation of DNA errors and thus fixed mutations during cell proliferation by 100-1000 fold [343].

There are 7 major genes which encode for enzymes involved in MMR. They include the MutS-homologs (MSH2, MSH3 and MSH6) and the MutL homologs (MLH1, MLH3, PMS1 and PMS2). Microsatellites are multiple tandem repeats of 1-6 nucleotides distributed within the genome. These are vulnerable to insertion/deletion mutations and defects in any one of the MMR genes causes an abnormal accumulation of microsatellites repeats resulting in MSI. MSI in oncogenes or tumour suppressor genes increase susceptibility to damaging frameshift mutations resulting in a truncated, non-functional protein or loss of protein, both which drive tumorigenesis [343].

Deficiency in MMR (dMMR) can be caused by both genetic and epigenetic defects in any one of the MMR genes. In germline dMMR, the wild-type allele can be inactivated via somatic nonsense mutations, LOH or by methylation, resulting in the second 'hit' in hereditary cancers. In sporadic cancers due to somatic dMMR, the most common mechanism is via MLH1 promoter hypermethylation, and less commonly, biallelic inactivation of MMR genes. In colorectal cancers, MLH1 promoter methylation correlates with BRAF V600E mutations and negatively predicts for germline MMR mutation status [345].

Lynch syndrome (LS) is an autosomal dominant disease caused by germline MMR gene mutations which cause several malignancies associated with younger age of onset [346]. 90% of LS are due to germline mutations in MLH1 and MSH2, whilst MSH6 and PMS2 are much rarer, occurring in 7-10% and <5% of cases, respectively [346]. Germline mutations in PMS1, MSH3 and MLH3, are very rare [347]. Colorectal and gynaecological cancers are the two most common LS associated cancers [348]. In LS, the cumulative lifetime risk of colorectal cancers is 50-70% [348], 40-60% for endometrial cancer, and 12%-17% risk for EOC [346, 348]. Other associated malignancies include upper gastrointestinal, urothelial, prostate and brain tumours. The age of onset and cumulative lifetime risk of these cancers also differs according to each germline MMR gene mutation [349]. In the largest prospective observational study published of 3119 women, the cumulative incidence at 75 years for LS associated gynaecological cancers was 10.1%, 16.9%, 13.1% and 0% for ovarian cancers, and 42.0%, 56.7%, 46.2% and 26.4% for endometrial cancers, in MLH1, MSH2, MSH6 and PMS2 carriers, respectively [348]. The age of onset was more likely to be under 40 years of age for MLH1 and MSH2 mutations, and over 40 years in those with MSH6 and PMS2 mutations. Similar findings were demonstrated in another large retrospective study of 1063 patients [350]. Furthermore, women with truncating MLH1 mutations developed endometrial cancer at an older age than those with non-truncating mutations

(P=0.002) in this study [350]. Together, these studies conclude that cancer surveillance for patients with LS could potentially be tailored according to type of mutation.

Both MMR IHC and MSI testing are useful surrogate screening tools for dMMR tumours. The biological rationale and interpretation of MMR IHC is discussed in detail in section 2.5.1.2. MSI is evaluated from a panel of five validated microsatellites [351]. They include two mononucleotides BAT25 and BAT26, as well as three dinucleotides, D2S123, D5S346 and D17S250. MSI status is then classified as MSI-high (at least two unstable markers), MSI-low (one unstable marker), and microsatellite stable (MSS) (no unstable markers). In colorectal cancer, MSI testing and MMR IHC demonstrate good concordance and has an overall reported sensitivity and specificity of more than 90% [351].

Approximately 30% of endometrial carcinomas and 18% of colorectal carcinomas are dMMR. Most are somatically acquired although 6% and 3% are due to LS, respectively [352]. In colorectal cancers, the Amsterdam II and revised Bethesda guidelines based on age, personal and family history were developed to help identify patients with LS [353]. However, universal MMR testing in colorectal carcinomas is now recommended due to the prognostic and predictive significance of detecting dMMR [354]. This practice is similarly starting to emerge for endometrial carcinomas in North American centres but has not been established worldwide [355]. In both colorectal and endometrial carcinomas, dMMR is associated with improved prognosis over MMR proficient (pMMR) tumours [353, 356]. In colorectal cancers, dMMR predicts for chemotherapy resistance to 5-flurouracil [357], although this association is less established in endometrial carcinomas. Furthermore, there is now emerging data suggesting that MSI-high tumours have better responses to immune checkpoint inhibitors [358]. On this basis, the United States Federation of Food and Drug Administration recently granted accelerated approval for pembrolizumab, an immune check point PD1 inhibitor, for all MSI-high or dMMR tumours. Tumour mutation burden (TMB) is also associated with MSI status. MSI-high tumours are usually hyper-mutated due to dMMR, ranging from 10 to 100 mutations per Mb, whereas MSS tumours exhibit lower mutation frequencies between 1 to 10 mutations per Mb. TMB correlates with the level of neo-antigens thus increasing the immunogenicity of tumours [359]. High TMB (approximately above 10 mutations per Mb) is increasingly recognised as a predictive biomarker for immune checkpoint inhibitors, although the exact thresholds for different malignancies has not been formally established [359]. Emerging work is also beginning to identify MSS tumours with high TMB who also derive benefit from immunotherapy [359].

1.5.8.5.1 Mismatch repair deficiency in EnOC

Whilst dMMR in colorectal and endometrial carcinomas has been extensively investigated, data on dMMR in EOC is relatively sparse. Overall, 2%- 29% of EOC display dMMR by loss of IHC expression, and 5-15% display MSI [343]. Meta-analyses performed by Pal et al and Murphy et al finds the pooled proportion of MSH-high EOC to be 10-12% [347, 360]. Germline dMMR is rare and only accounts for 1-2% in EOC [343, 361].Some studies performed to date have found dMMR EOC correlates with the MSI-high phenotype [362-364], whereas others have demonstrated poor concordance [365, 366]. dMMR occurs most commonly in non-HGSOC tumours, with highest frequencies observed in EnOC followed by CCOC [361, 367, 368]. In contrast, very low frequencies of one percent or less have been observed in HGSOC [352, 369, 370]. LS associated EOC tends to occur in younger women (median 45 years) of non-serous histology with early stage disease and good prognosis. Amongst patients with LS, the highest frequencies of EOC are observed in EnOC (19%-35%) followed by CCOC (12%-17%) [346, 367, 371]. In line with this, the most recent College of American Pathologists guidance now recommends universal MMR screen for EnOC and CCOC. This practice is however not established worldwide.

In contrast to endometrial carcinomas, the frequency of dMMR (by IHC) in EnOC is lower at 7-14% [255, 302, 352, 361, 372]. The prognostic role of dMMR in EnOC is less certain. Rambau et al evaluated MMR status in 104 pathology reviewed EnOC. In this study, the prevalence of dMMR was 13.8% [352]. Cases with loss of MSH2/MSH6 were more likely to present at a younger age compared to those who were MMR proficient (44.4 years versus 54.8 years; P=0.0024), whereas no differences were observed in age of onset for patients with loss of MLH1/PMS2. Cases with MSH2/MSH6 loss had higher CA125 levels at diagnosis, whereas MLH1/PMS2 loss cases were more likely to present with higher grade disease and synchronous EnEC. Overall, no differences between DSS and RFS was observed between dMMR and MMR proficient tumours in EnOC of all grades and stages. In contrast, Parra Herran et al found in a cohort of 97 pathological reviewed EnOC, tumours with dMMR had superior outcomes independent of grade and stage [302].

A few studies have also evaluated the association between dMMR EnOC and histo-pathological features in order to guide MMR testing. In Aysal et al, they found the lack of an adenofibromatous background was predictive of MMR loss, however peritumoral lymphocytes and tumour infiltrating lymphocytes were rare in 71 EnOC [373]. This low frequency was also confirmed by Chui et al in 20 LS associated ovarian carcinomas, of which 14 were EnOC [374]. This finding contrasts with those of Rambau et al which found that at least 20 CD8 positive TILs per high powered field (HPF) was independently associated with dMMR although reported poor sensitivity and specificity of this test [352]. Bennet et al found TILs to average 47/10 high powered fields with no association of clinico-pathological features with dMMR [372]. These studies conclude that universal reflex MMR testing of EnOC may be warranted. The immunogenicity of EnOC was explored in the study by Wang et al which performed whole genome sequencing of 29 EnOC. Here, of the third of tumours which displayed MSI, higher number of immunogenic epitopes were observed compared to MSS cases (P=0.0023) [375]. This

suggested greater neo-antigen generation in these cases, and the authors conclude that EnOC may be candidates for immune checkpoint inhibitors.

1.5.8.6 Molecular stratification of endometrial carcinomas and EnOC

The landmark endometrial carcinoma TCGA study performed multi-platform analysis incorporating somatic copy number alterations (CNA), single nucleotide variants and TMB. It identified 4 molecular groups which were: i) *POLE*-mutant, ii) MSI-high (MSI-H), iii) copy number low (CN-low) and iv) copy number high (CN-high; serous like) [356]. Each molecular group demonstrated distinct genomic aberrations and differing survival outcomes. The *POLE*-mutant group displayed the best PFS of the four groups. This ultra-mutated copy number stable group had recurrent mutations in the exonuclease domain of DNA polymerase episilon (*POLE*) and was characterised by an extremely high somatic mutation burden of greater than 100 mutations per megabase. Notably, tumours in the *POLE*-mutant group also demonstrated diverse morphologies. The MSI-H group was a hyper-mutated group with mutations in MMR genes and high mutational frequencies of greater than 10 mutations per Mb. This group comprised predominantly endometrioid histologies. The CN-low group was MMR proficient and characterised by genomic stability with mutations in the PI3K/AKT and Wnt signalling pathways. This group was exclusively made up of EnEC with high expression of ER and PR. The CN-high group demonstrated high somatic CNA with frequent *TP53* mutations and displayed the worst PFS (Figure 2) [356].

The PROMISE algorithm was developed and proposed as a more pragmatic algorithm stratifying endometrial carcinomas into the four prognostic groups, outcomes which mirror that of the pivotal TCGA study (Figure 3) [376]. When it was applied to the intermediate and intermediate-high risk early stage patients as part of the PORTEC 1 and 2 clinical trials, integrated use of the PROMISE algorithm with traditional clinico-pathological factors resulted in improved risk stratification [377, 378]. Those with *POLE* mutant endometrial carcinomas had no loco-regional recurrences whereas those with p53 mutant IHC expression had a 6 fold increase in loco-regional recurrences when compared to those with p53 wild-type IHC expression [378].

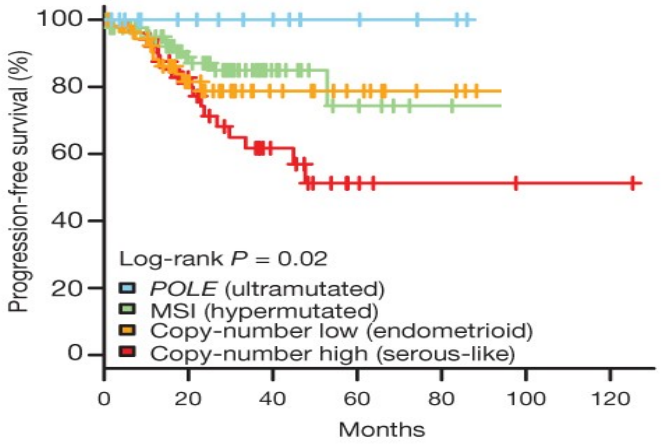

Figure 2: Progression free survival of molecular groups identified from the endometrial TCGA.

Figure from Levine et al, Nature 2013 [356].

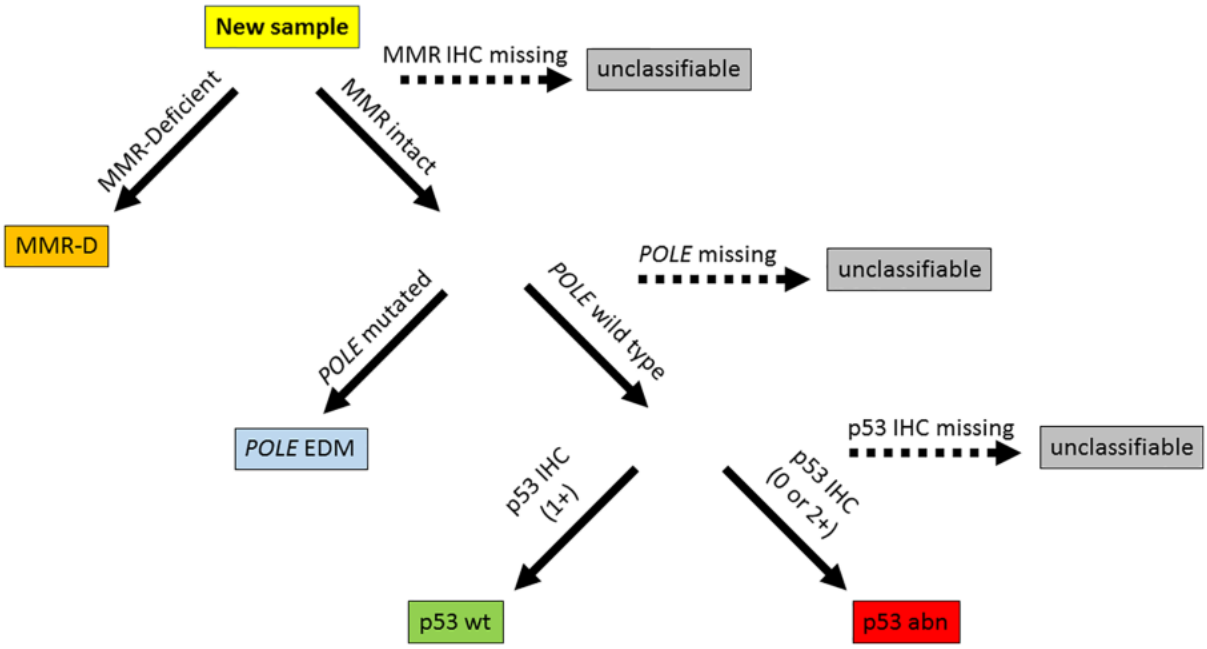

Figure 3: PROMISE algorithm reflecting the molecular groups identified in the endometrial TCGA. The first step utilised mismatch repair (MMR) immunohistochemistry (IHC) to identify MMR deficient tumours, this is followed by testing for *POLE* exonuclease domain mutations (EDM), and finally for aberrant p53 expression on IHC. Figure from Talhouk et al, Cancer 2017 [376].

The findings of the endometrial TCGA study prompted a similar analysis in EnOC. Parra-Herran et al applied the PROMISE algorithm to 72 contemporary pathology reviewed ovarian EnOC [302]. In this study, only WT1 negative samples with at least one confirmatory endometrioid feature were included. The five year RFS of the three main prognostic groups in this cohort were 100% in the *POLE* mutated (n=7; 10%) and dMMR tumours (n=6; 8%), 82% and 42% in the p53 wild-type (n=42; 58%) and p53 mutant expression groups (n=17; 24%), respectively. The molecular classifier was found to be an independent prognostic factor on multivariable analysis which accounted for stage and grade, but not residual disease [302].

Three studies have performed whole exome and whole genome sequencing of EnOC. In Teer et al, a cohort of non-serous tumours underwent WES and target gene sequencing of 1321 genes [379]. These included six pathology reviewed stage I EnOC (three grade 1 and three grade 2) for WES, and 14 EnOC (grade not reported) for targeted sequencing. The frequencies of common EnOC mutations were similar to that described earlier (Figure 4). Notably, *TP53* mutations were in six of the 14 EnOC which underwent target gene sequencing but none in the WES cohort. Across both sequenced cohorts, *BRCA*2 mutations were identified in 5 EnOC (two truncating mutations, three missense mutations). All five of these tumours also had concurrent mutations in at least one classical EnOC genes (*ARID1A*, *PIK3CA*, *PTEN*, *CTNNB1*). The authors suggest that the findings of mutations in HRR genes may suggest that these tumours may respond to PARP inhibitors [379].

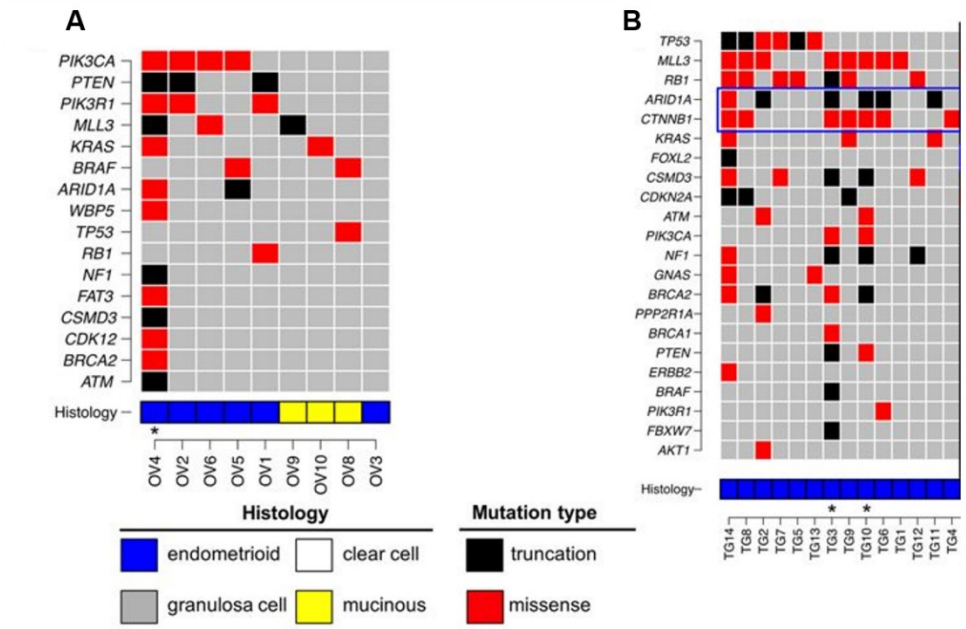

Figure 4: (A) Mutation profiles of six stage I grade 1 and 2 EnOC identified through whole exome sequencing analysis of paired tumour and normal samples. (B) Mutation profiles of 14 EnOC (unknown grade) identified through target gene sequencing. Asterixes indicate *POLE* proofreading domain mutations. Figure from Teer et al, Scientific Reports 2017 [379].

In Wang et al, 133 ovarian tumours, which included 29 EnOC and matched normal samples, underwent whole genome sequencing and copy number analysis [375]. Information on grade or whether pathology review of these samples were performed were not available. Seven molecular groups were identified between and within each histological subtype. These groups were: i) G-BC (granulosa cell tumours with a mutation signature associated with breast cancer and medulloblastoma), ii) E-MSI (EnOC with a mutation signature of dMMR), iii) Mixture (HGSOC, CCOC and EnOC with no discriminant features), iv) C-APOBEC (CCOC with a mutation signature defined by activity of the AID/APOBEC family of cytodine deaminase), v) C-AGE (CCOC with a mutation signature associated with age of diagnosis); vi) H-FBI (HGSOC with a high frequency of fold-back inversions structural variants); and vii) H-HRD (HGSOC with frequent duplications or deletion rearrangements and characterised by a mutation signature of HRD). Within EnOC, three main molecular groups were identified (Figure 5). The first was the ultra-mutated group, characterised by a *POLE* mutation signature. The E-MSI group, in which a third of tumours clustered within, contained no focal CNAs with RPL22 mutations in 50% of the group. The MSS group, in which 40% contained *TP53* mutations, lacked a strong genomic signature and clustered across the 6 molecular groups. Notably, 14% of EnOC in this study clustered in the H-HRD group, a finding similar to that of Teer at al. The MSS group also contained the highest proportion of *CTNNB1* and *KRAS* mutations (Figure 6) [375, 379]. Interestingly, the number of immunogenic epitopes were higher in the E-MSI group compared to those in the MSS group (P=0.0023), suggesting that these tumours may derive benefit from immune checkpoint inhibitors [375, 380].

Cybulska et al investigated the mutational landscape of EnOC without concurrent synchronous endometrial carcinomas and compared this to EnOC with synchronous endometrial carcinomas, HGSOC, and EnEC [381]. Eight tumours (all grades of EnOC) underwent massively parallel sequencing targeting 341-468 cancer related genes and the remaining 28 EnOC (unknown grade) underwent WGS (derived from the study performed by Wang et al [375]) (Figure 7). Here, the most commonly mutated genes were *KRAS* (42%), *PIK3CA* (39%), *PTEN* (33%), *CTNNB1* (25%) and *ARID1A* (19%). 17% of tumours harboured *TP53* mutations, whilst 19% were MSI-high. *POLE* mutations were infrequent (3%). Similar to Teer et al [379], 11% of EnOC harboured *BRCA*2 non-synonymous mutations (three missense and one truncating mutation) [381].

A formal comparison of 341 cancer-related genes was performed between pure EnOC with MSI-high and *POLE* exonuclease domain mutated EnOC removed, and HGSOC from TCGA [131, 381]. Here, *KRAS, PIK3CA, PTEN* and *PIK3R1* were significantly more frequent in non-hyper-mutated pure EnOC compared to HGSOC, whereas *TP53* was more significantly more frequent in HGSOC. When compared to EnEC from TCGA, similar genes were mutated albeit at different frequencies. *PTEN, PIK3R1, ARID1A, KMT2D* and *CTCF* were more commonly mutated in EnEC than pure EnOC, whereas *KRAS* and *PIK3CA* mutations were present at similar frequencies [381]. These differences held true when comparing pure EnOC to EnOC with synchronous endometrial carcinomas, consistent with the emerging data suggesting that EnOC with synchronous endometrial carcinomas are clonally related [236, 382]. Due to a higher proportion of grade 2 and grade 3 EnEC in the TCGA cohort, a matched

analysis with grade 1 and 2 EnEC was performed. Here only *PTEN* mutations were significantly lower in EnOC compared to EnEC [381].

The PROMISE algorithm was subsequently applied to this cohort of tumours and all four molecular subtypes were identified. The *POLE* mutant (3% versus 11%) and MSI-high cohorts (19% versus 34%) in EnOC were numerically less frequent, whilst the p53 wild-type expression (copy-number low) (61% versus 47%), and p53 aberrant expression (copy-number high) (17% versus 9%) cohorts were more frequent, when compared to the corresponding surrogate cohorts in the endometrial TCGA. These differences were however not statistically significant. Furthermore, no significant differences in clinical outcome between the PROMISE cohorts in pure EnOC were observed.

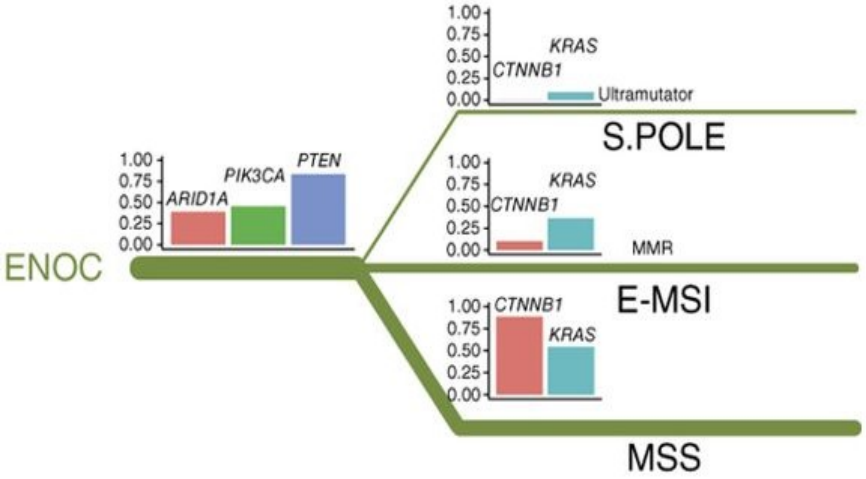

Figure 5: Molecular subgroups of EnOC.

Figure from Wang et al, Nature 2017 [375]. S.*POLE*= *POLE* mutations signature; E-MSI= microsatellite unstable tumours with a mismatch repair deficient mutation signature; MSS= microsatellite stable tumours.

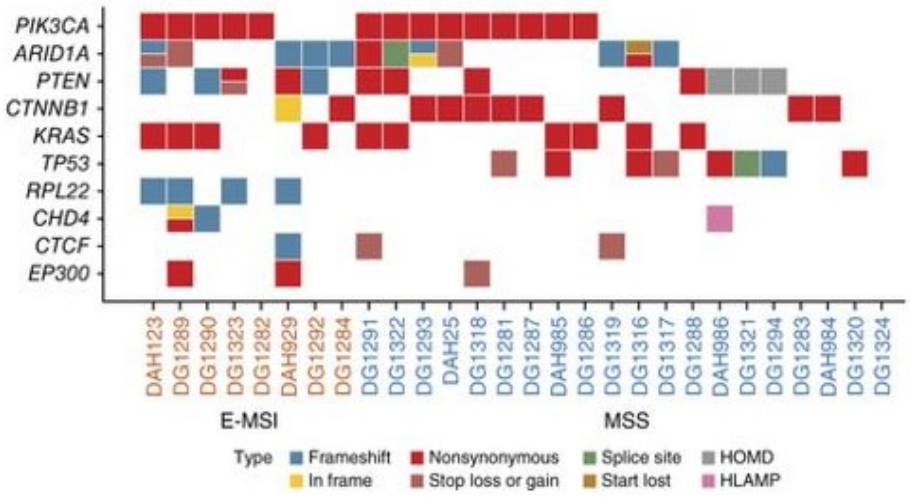

Figure 6: Oncoplot displaying frequent mutations in EnOC in MSI-high versus MSS tumours.
Figure from Wang et al, Nature 2017 [375].

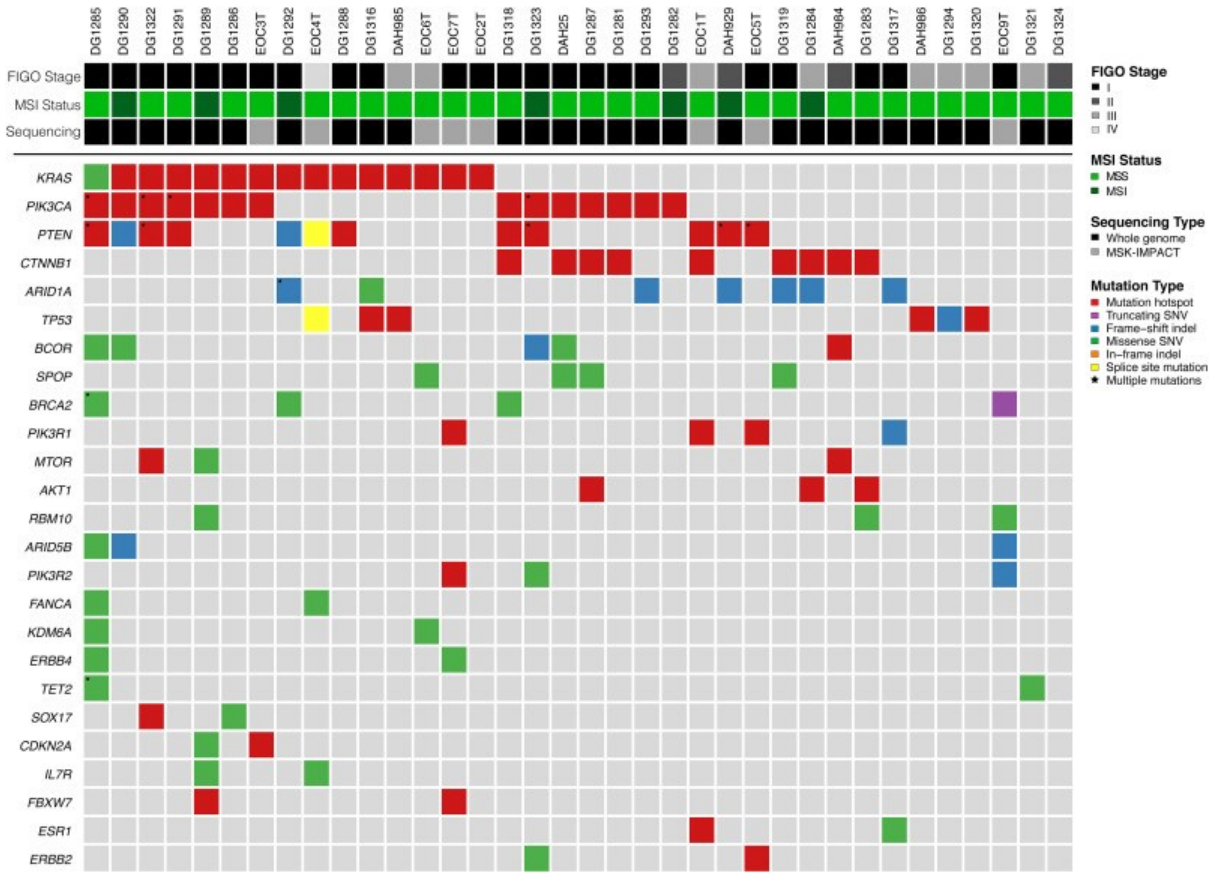

Figure 7: Oncoplot displaying non-synonymous mutations identified following whole genome sequencing (n=28; black box) and massively targeted parallel sequencing (n=8; grey box) identified in EnOC.
Figure from Cybulska et al, Gynecologic Oncology 2019 [381].

These three studies investigating the molecular landscape of EnOC demonstrates that it is a molecularly heterogeneous disease. However, all of these studies have analysed small patient cohorts which have lacked the statistical power to correlate molecular events to patient outcome. Furthermore, whether high grade EnOC constitutes a unique entity both clinically, pathologically and at the molecular level, or simply a pathological variant of HGSOC, continues to remain elusive.

1.5.9 Aims and hypothesis

The hypothesis of this study is that there are pathological and molecular subgroups of endometrioid ovarian carcinoma that correlate with clinical outcome.

The objective of this study is to answer the following clinical and scientific questions:

- How do WT1 negative EnOC behave as a clinical entity?
- What are the predominant genetic mutations that occur in EnOC?
- Are there any mutationally defined molecular subgroups within low and high grade EnOC and how do they correlate with clinical outcome?
- What is the prognostic and predictive value of ER, PR and AR expression in EnOC?
- Are low and high grade EnOC different diseases at the clinical and genomic level, and should a two-tier grading system be used instead?

2. Materials and Methods

2.1 Ethical approval

Pathology samples obtained before 1st September 2006 are automatically covered by the Human Tissues Act Scotland 2006. All subsequent human tissue specimens used for research have ethical approval obtained from South East Scotland Scottish Academic Health Sciences Collaboration (SAHSC) BioResource (reference: 15/ES/0094-S494). Correlation of molecular data to clinical outcome and clinico-pathological variables in ovarian cancer was approved by NHS Lothian Research and Development (reference 2007/W/ON/29).

2.2 Pathology review

Between August 1968 and May 2014, 505 patients were identified through the Edinburgh ovarian cancer database with a diagnosis of EnOC or mixed tumours with an endometrioid component. 234 of these cases were not evaluable, with the majority (n=216) due to irretrievable tumour blocks. Of the remaining 271 cases, the most representative sample from the primary tumour site from corresponding pathology reports and pre-existing haematoxylin and eosin (H&E) slide series (n=89) were identified. Only baseline chemotherapy naïve samples were included due to the recognised effect of chemotherapy on morphology [383]. Six tumours were identified as endometrial cancer metastases (n=6) and excluded. Of the remaining 265 tumours, six 5μm sections were taken from the selected archival formalin fixed paraffin embedded (FFPE) tumour block and stained for H&E WT1 (DAKO, clone 6F-H2; 1:1000 dilution) and p53 (DAKO, clone DO-7; 1:50 dilution) (details in section 2.3).

Pathology review was conducted as per WHO 2014 classification by an expert gynaecological pathologist (CSH). The presence of endometriosis (Figure 9B) or synchronous endometrial carcinomas were recorded either from the reviewed slide/s or if mentioned in the pathology report. Following WT1 and p53 immunohistochemistry (IHC), tumours excluded were: 1) WT1 positive HGSOC (n=109), 2) WT1 positive mixed grade 1 and 3 EnOC (n=1), 3) tumours of other histology (n=19), and 4) tumours with non-evaluable clinical outcome data (n=2) (Figure 8). Six WT1 positive p53 wild-type expression grade 1 and 2 EnOC were also identified and formed a separate cohort of interest (Figure 8).

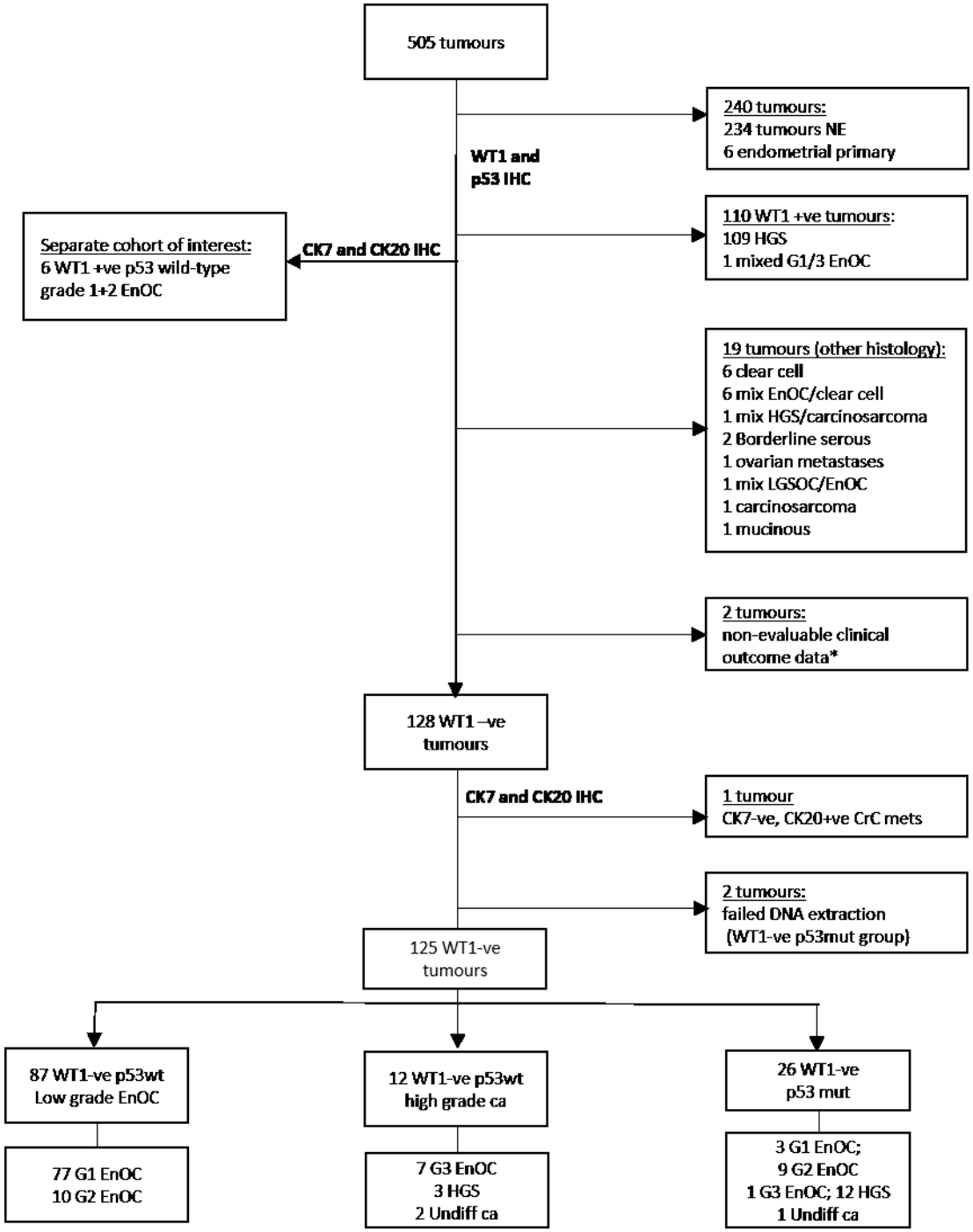

Figure 8: Consort diagram of the pathology review process.

Legend: NE=not evaluable; IHC=immunohistochemistry; +ve=positive; -ve=negative; HGS=high grade serous morphology; EnOC=endometrioid; LGSOC=low grade serous; CrC=colorectal carcinoma; mut=mutant; met=metastases; Undiff=undifferentiated carcinoma. *Due to concurrent metastatic breast cancer.

CK7 (Leica, Clone RN7, 1:100 dilution) and CK20 immunostaining (Leica, clone KS20.8, 1:50 dilution) was performed on the remaining 128 WT1 negative tumours and the six WT1 positive low grade EnOC to exclude colorectal metastases (n=1 identified) (details in section 2.3.2). The remaining 127 WT1 negative tumours were assigned to three IHC cohorts based on p53 expression pattern and tumour grade:

- **Group 1:** WT1 negative, p53 wild-type expression, low grade EnOC (n= 87) (grade 1 (Figure 9C), grade 2 (Figure 10A))
- **Group 2:** WT1 negative, p53 mutant expression carcinomas (low grade EnOC and high grade carcinomas) (n=28)
- **Group 3:** WT1 negative, p53 wild-type expression high grade carcinomas (n=12).

WT1 negative high grade carcinomas in this study included those with the histological appearances of grade 3 EnOC (Figure 10B), HGSOC (Figure 11A), and undifferentiated carcinomas (Figure 11B), due to the recognised morphological overlap displayed by these tumours. As discussed extensively in chapter 1, WT1 negativity is a useful and validated marker in distinguishing HGSOC from grade 3 EnOC [229]. Undifferentiated carcinomas were also included due to the association with low grade EnOC as distinct de-differentiated carcinomas with emerging data to support a clonal relationship between each entity [258].

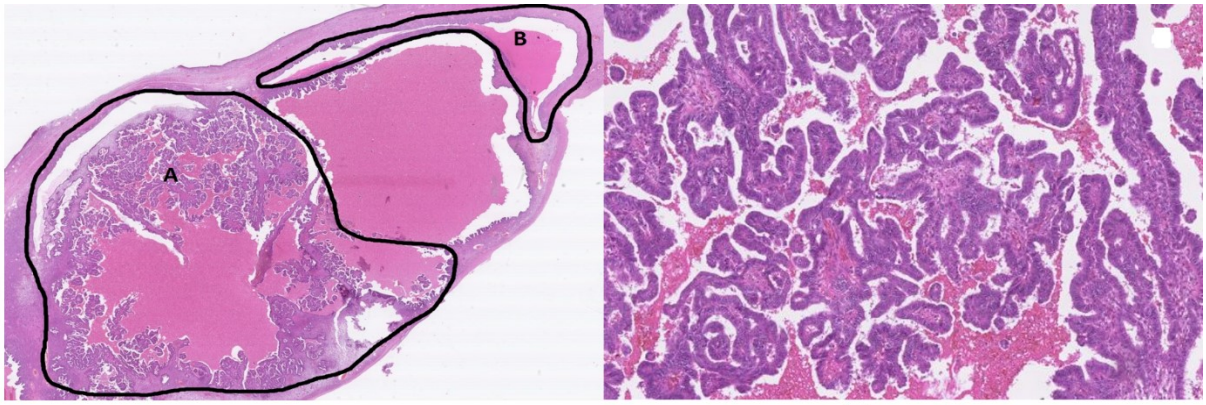

Figure 9A and 9B

Figure 9C

Figure 9: H&E stain of grade 1 EnOC (A) arising from adjacent endometrioisis (B). H&E stain of grade 1 EnOC (C).

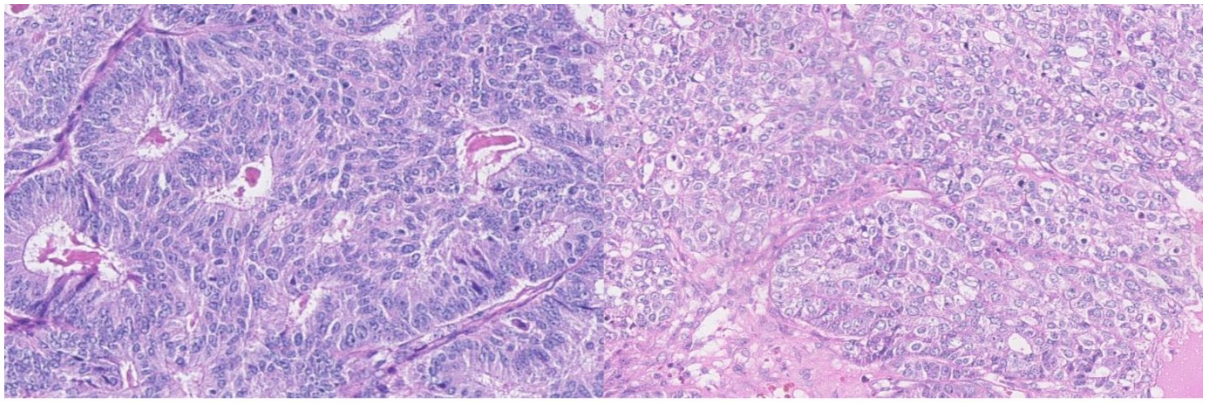

Figure 10A

Figure 10B

Figure 10: H&E stain of grade 2 (A) and grade 3 (B) EnOC.

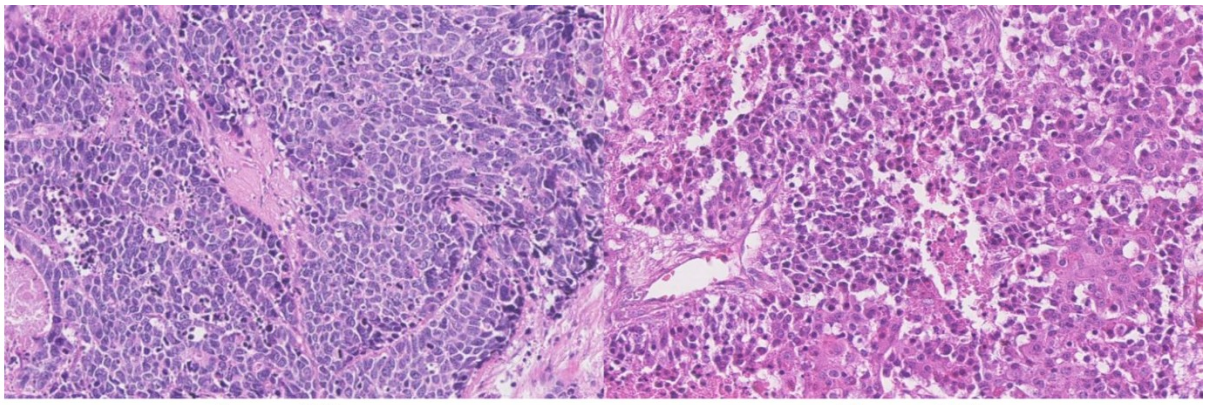

Figure 11A

Figure 11B

Figure 11: H&E stain of WT1 negative carcinoma with high grade serous morphology (A), and WT1 negative undifferentiated ovarian carcinoma (B).

2.3 Immunohistochemistry

2.3.1 WT1 and p53 IHC staining

Both IHC staining for WT1 and p53 were performed on the Leica Bond III machine as per protocol F. WT1 staining was performed using a 1:1000 dilution of the monoclonal mouse anti-human WT1 antibody clone 6F-H2 (DAKO) (antigen retrieval Bond solution 2: 20 minutes). Tumours with any WT1 nuclear positivity were considered WT1 positive (Figure 12(i)), while complete absence of nuclear WT1 with internal control vascular positivity was considered WT1 negative (Figure 12(ii)). p53 IHC staining was performed using a 1:50 dilution of the monoclonal mouse anti-human p53 antibody cloneDO-7 (DAKO) (antigen retrieval Bond solution 1: 20 minutes). A tissue section of HGSOC showing both WT1 positivity and aberrant diffuse (mutant) p53 positivity served as a control for both IHC stains.

P53 IHC staining was evaluated according to three staining patterns. Two patterns have been shown to correlate with a *TP53* mutation [384, 385]. The first is a strong and diffuse nuclear stain pattern indicative of missense mutations; this was recorded as 'aberrant diffuse' (Figure 13(i)). The second pattern is complete lack of nuclear staining which is associated with nonsense mutations; this was recorded as 'null' (Figure 13(ii)). Both aberrant diffuse and null patterns were termed p53 mutant expression. The third pattern is variable nuclear expression of the p53 immunostain due to the relative instability of the protein due to its short half-life in p53 wild-type samples [385]. This was recorded as 'p53 wild-type' (Figure 14). Positive nuclear staining of stromal cells served as internal controls and was an absolute requirement for identification of the 'null' pattern. Tumours which exhibited cytoplasmic staining of p53 were evaluated based on the nuclear staining pattern as outlined above.

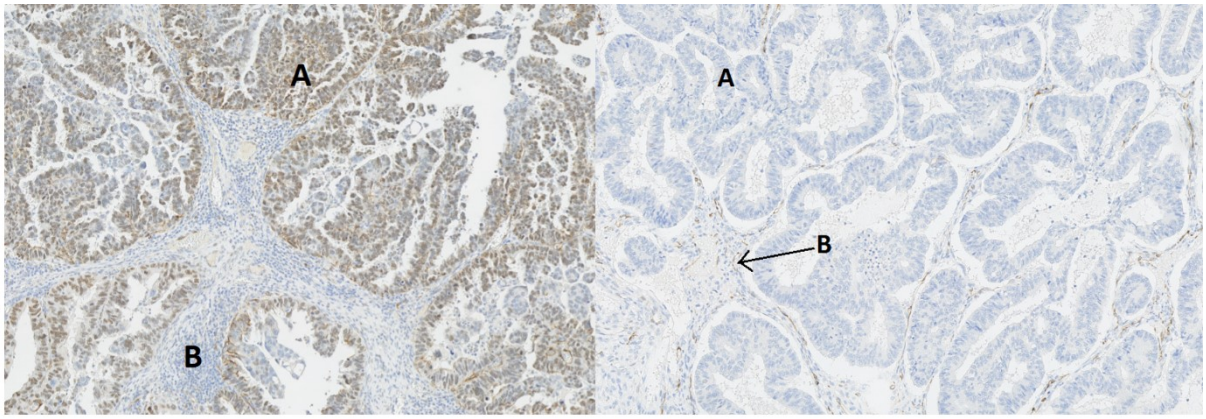

Figure 12(i) **Figure 12(ii)**

Figure 12(i): Diffusely strong nuclear positive WT1 immunohistochemistry stain (A) with negative non-vascular stroma (B). Figure 12(ii): Negative WT1 immunohistochemistry stain (A) with positive vascular endothelial cells as an internal control (B).

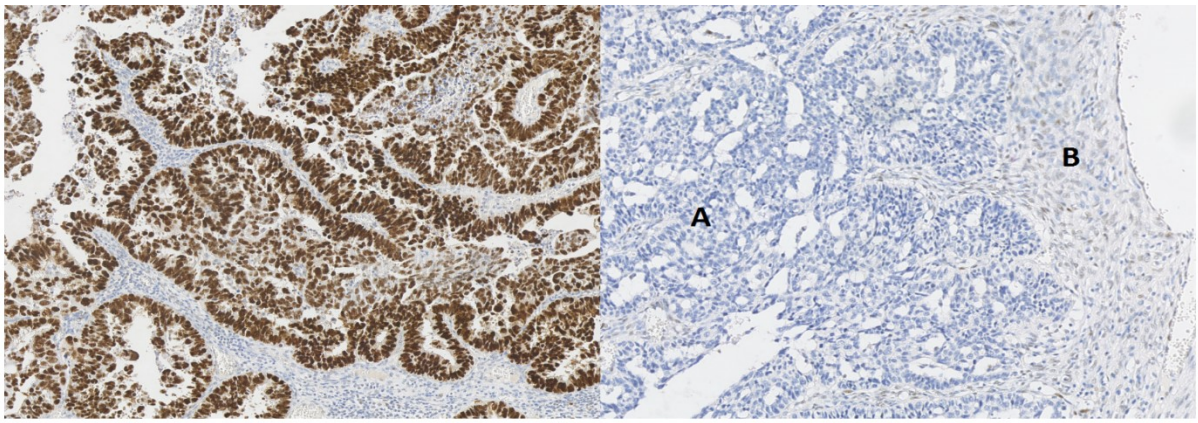

Figure 13(i) **Figure 13(ii)**

Figure 13(i): Aberrant diffuse p53 immunohistochemistry stain. Figure 13(ii): Null p53 immunohistochemistry stain (A) with positive stroma (B) as an internal control.

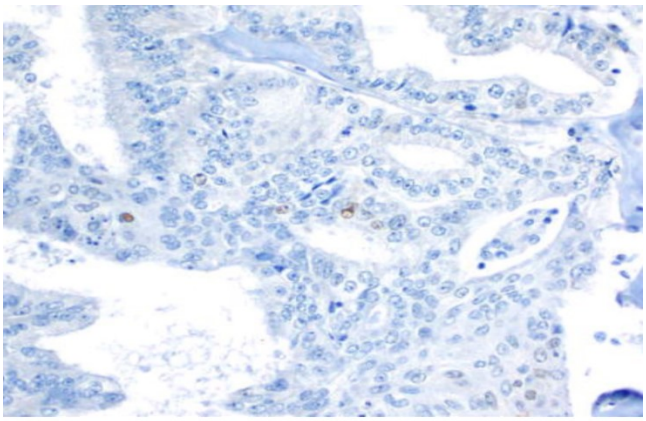

Figure 14

Figure 14: Wild-type p53 expression on immunohistochemistry with variable nuclear staining.

2.3.2 CK7 and CK20 immuno-staining

All samples in the WT1 negative primary cohort and the WT1 positive low grade EnOC cohort underwent CK7 (Figure 15A) and CK20 immunostaining (Figure 15B). Samples which were CK7 negative and CK20 positive were diagnosed as likely colorectal metastases and excluded. Antibody staining was performed using the Leica Bond III autostainer using Protocol F.

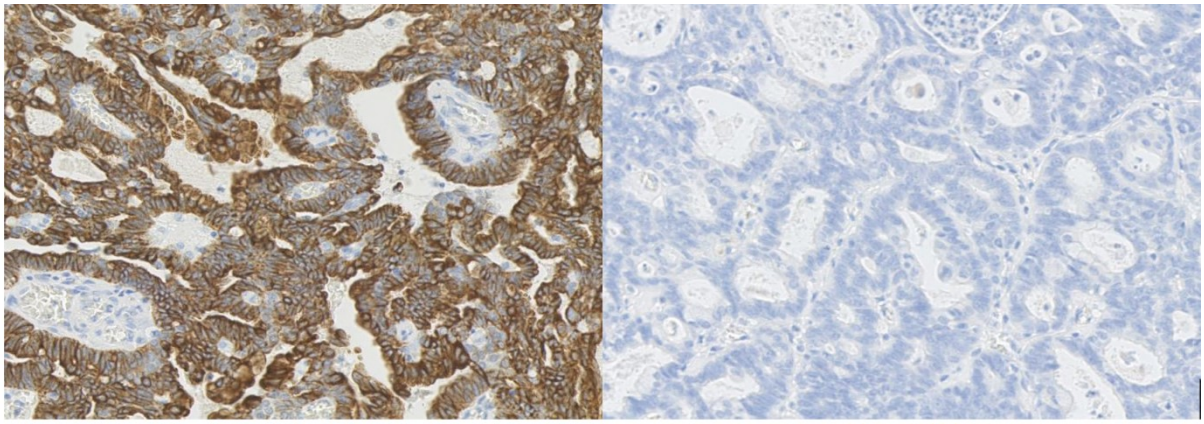

Figure 15A Figure 15B

Figure 15: CK7 positive immunohistochemistry (A), CK20 negative immunohistochemistry (B).

CK7 staining was performed using a 1:100 dilution of the monoclonal mouse CK7 antibody (Leica, Clone RN7, Hier1 – 20 minutes). A WT1 positive p53 aberrant expression HGSOC tissue section was used as a control. CK7 staining was considered positive with any positive nuclear staining of tumour cells.

CK20 staining was performed using a 1:50 dilution of the monoclonal mouse CK20 antibody (Leica, clone KS20.8, Hier1-20 minutes). Normal stomach tissue was used as a control. CK20 staining was considered positive with any positive nuclear staining of tumour cells.

2.4 Clinical data extraction

Clinico-pathological variables and outcome data were prospectively entered into a database for each patient as part of routine care. Baseline characteristics including age, date of diagnosis, date and cause of death, stage at diagnosis, residual disease following surgery and details of surgical staging were extracted from the Edinburgh Ovarian Cancer database. Due to the variable definitions used to record data over time, I manually reviewed all patient case notes to ensure a consistent approach was used. In particular, all patients with a residual disease recorded as <2cm were specifically reviewed. Cases with a clinical annotation or surgical note stating complete cytoreduction was achieved were recorded as 0cm. Full surgical staging was defined in my study as total abdominal hysterectomy, bilateral salpingo-oophorectomy, omental biopsy or omentectomy, and washings. Patients who underwent systematic lymph node sampling or lymphadenectomy were recorded. Patterns of first relapse for stage I and II disease and sites of disease at presentation for stage IV patients were also recorded.

Data capture was censored as of 4th September 2017. DSS was recorded as the duration between the date of diagnosis and date of death from ovarian cancer. RFS was recorded as the duration between the date of diagnosis and the date of first radiological progression or recurrence, or death from EnOC.

Treatment characteristics including type, line of treatment, indication, duration of therapy, and reasons for treatment cessation were recorded. Patients were evaluable for radiological responses if baseline radiological imaging was performed within six weeks of the start of therapy with measurable disease, and had follow up radiological imaging during their treatment following at least 3 cycles of platinum based chemotherapy. As many patients did not have measurements on their radiology reports, best radiological response was documented as per the reporting radiologist across the duration of therapy. In order to prevent overestimation of radiological response rates, only patients with at least a 30% decrease in the sum of measurements provided on the report were considered to have a partial response (PR). Patients with complete response (CR) or progressive disease (PD) were recorded as per the reporting radiologist. If the radiologist reported a response with no measurements provided, they were recorded as stable disease (SD). Patients who had complete macroscopic cytoreduction with no baseline imaging who then developed new metastases after at least 3 cycles of platinum based chemotherapy were considered to have PD.

The best CA125 response to platinum based chemotherapy across the duration of therapy was also recorded. Due to the variable frequency of CA125 measurements, a modified GCIG criteria was adopted [386]. Patients were evaluable for CA125 response if they had an evaluable CA125 (>70U/ml) within four weeks of starting therapy, and had at least a further 2 CA125 responses recorded, with the last value within 4 weeks of completion of treatment. A second CA125 value at least 4 weeks after a CA125 CR or PR was required as confirmatory evidence. Patients who received post-operative platinum based chemotherapy following complete macroscopic cytoreduction were not considered evaluable for CA125 response due to the effect of surgical cytoreduction on CA125. Patients with residual disease of more than 2cm post primary or secondary cytoreductive surgery were evaluable for CA125 responses.

In patients who received endocrine therapy, CA125 was most commonly used by clinicians as a marker of response. Radiology was only performed when there was evidence of a significant rise in CA125 or symptomatic deterioration. As such, radiological PFS could not be accurately defined in this study. Endocrine therapy was continued until there was symptomatic disease progression prompting further chemotherapy. Thus, the duration of therapy was recorded as an objective end-point and surrogate of endocrine sensitivity.

In addition to the CA125 evaluability criteria for those receiving platinum based chemotherapy as outlined above, patients were evaluable for CA125 response if they received endocrine therapy as treatment for at least four weeks duration, and if they had at least three CA125 values if they received endocrine therapy for greater than 12 weeks. If endocrine therapy was received for 12 weeks or less, two CA125 values were required with the second CA125 within 4 weeks of therapy cessation. Patients treated for less than 12 weeks but with clear CA125 progression were also considered evaluable. Patients with one CA125 measure, or if they received endocrine therapy for 12 weeks or less with no CA125 progression were not evaluable. The 12 week threshold was used as this has been shown to be the median time to a CA125 response to endocrine therapy [112].

The definitions of CA125 response were as per GCIG criteria. SD was defined if maintained for at least 12 weeks from treatment initiation. Progressive disease (PD) was defined as doubling of the baseline CA125 value. Response rates were calculated and recorded as follows:

- CA125 overall response rate (ORR)=CR+PR
- CA125 clinical benefit rate (CBR)= CR+PR+SD

The change in rate of rise of CA125 can demonstrate activity of cytostatic agents such as tamoxifen [387]. Stanley et al (Appendix C) described a group of patients whose CA125 rose (PD in 12 weeks or less by GCIG criteria) than stabilised (<50% rise in CA125) for at least another 12 weeks [295]. This delayed SD cohort was found to remain on endocrine therapy for a significantly longer duration than those who had disease progression by CA125 GCIG criteria but without the subsequent period of stabilisation (196 days versus 84 days, $P<0.0001$) [295]. Patients who demonstrated delayed CA125 SD on endocrine therapy in this study was also recorded.

2.5 Tissue Microarray construction

Tissue microarrays (TMA) were constructed from the WT1 negative primary cohort (n=125) and the WT1 positive low grade EnOC cohort (n=6) using a Mini Core machine. Six 0.8mm tissue cores were obtained from areas enriched for tumour using the marked H&E slide as reference. TMA triplicates were created for each patient sample and stained for 9 antibodies with appropriate controls. All IHC staining was performed on the Leica Bond III autostainer using protocol F.

2.5.1 TMA immunohistochemistry

All TMA IHC stain evaluation was performed initially by two independent observers (BS, YI) following a training session with CSH on each antibody. Cores with discordant scores were then reviewed together with a third observer (CSH) and a consensus score was obtained. All observers were blinded to the clinical outcome of each case.

2.5.1.1 Hormone receptors (ER, PR and AR)

ERα (referred to as ER from henceforth) staining was performed using a 1:50 dilution of the monoclonal rabbit anti-human ER antibody clone M3643 (DAKO, Clone EP1, antigen retrieval tris EDTA ph9, epitope retrieval solution (ERS 2) - 20 minutes). PR staining was performed using a 1:50 dilution of the monoclonal mouse anti-human PR antibody clone M3569 (DAKO, clone PgR 636, antigen retrieval Tris EDTA ph9, ERS2- 20 minutes). AR staining was performed used a 1:50 dilution of the monoclonal mouse anti-human AR antibody M3562 (DAKO, clone AR441, antigen retrieval Tris EDTA ph9, ERS2- 20 minutes). Normal breast tissue was used as the control for ER and PR, and normal prostate tissue for AR.

Hormone receptor stain interpretation

TMA cores were scored based on positive nuclear staining. The area of each core occupied by tumour was recorded as a percentage and the stain intensity was recorded as 1+ (weak positivity), 2+ (moderate positivity) or 3+ (strong positivity) as compared to a validated breast carcinoma control. Areas of squamous differentiation which are usually ER negative were also included. Cores which were missing or with less than 10% tumour were excluded. A weighted histoscore accounting for the percentage of tumour (% tumour) and stain intensity (1+, 2+ or 3+) was recorded for each core as follows: (% 1+ tumour x1) + (% 2+ tumour x 2) + (% 3+ tumour x 3) (Figure 16). If the difference between histoscores between BS and YI was less than 50 points, the histoscore was averaged. If the difference was 50 points or greater, the core was reviewed together with CSH, who scored it independently; the middle score was recorded. At least two cores had to have an evaluable weighted histoscore for the case to be considered.

The final histoscore for each case was calculated as follows:

$$\frac{(\text{core 1 Histoscore x A}) + (\text{core 2 Histoscore x B}) + (\text{core 3 Histoscore x C})}{A + B + C}$$

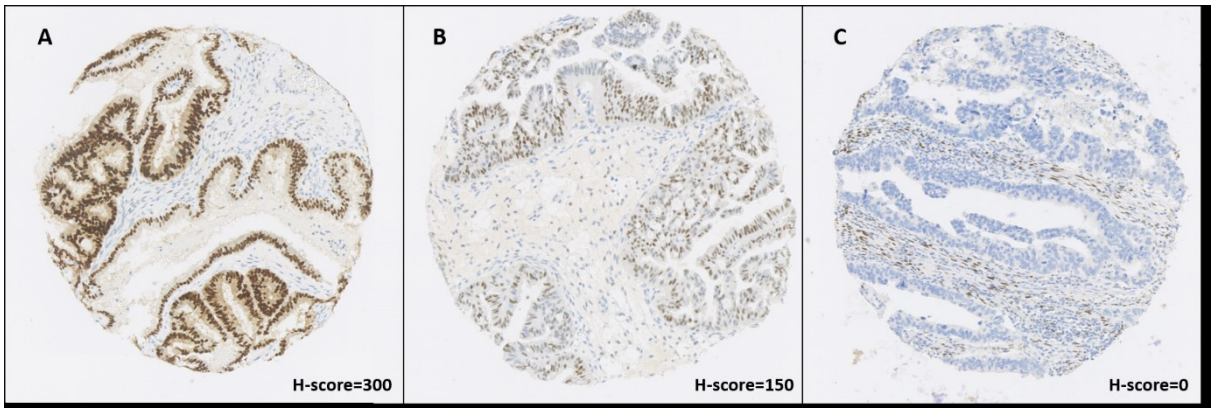

Figure 16: Examples of IHC stain interpretation for ER, PR and AR: A: histoscore 300, B: histoscore 150, C: histoscore 0 with positive stroma cells as an internal control.

2.5.1.2 Mismatch repair proteins

The MMR antibodies used were MLH1, MSH2, MSH6 and PMS2. These antibodies were chosen as they are used by the National Health Service and are thus clinically validated. Normal small bowel was used as a control for all four antibodies. MLH1 staining was performed using a 1:25 dilution of the monoclonal mouse anti-human MLH1 antibody M364001-2 (Agilent, clone ES05, ERS2 - 60mins). MSH2 staining was performed using a 1:75 dilution of the monoclonal mouse anti-human MSH2 antibody M363901-2 (Agilent, clone FE11, ERS2- 40 minutes). MSH6 staining was performed using a 1:25 dilution of the monoclonal mouse anti-human MSH6 antibody M364601-2 (Agilent, clone EP49, ERS1-30 minutes). PMS2 staining was performed using a 1:40 dilution of the monoclonal mouse anti-human PMS2 antibody M364701-2 (Agilent, clone EP51, ERS2-60 minutes).

MMR stain interpretation

For each core, any positive nuclear staining of tumour cells was recorded as '1' for that antibody. Complete loss of nuclear staining of tumour cells with positive stromal cells as an internal control was recorded as '0'. Cores which were missing or had absence of staining for both tumour and stromal cells were not evaluable. For each antibody, the case was considered 'intact' if at least 1 core was recorded as '1', and considered as 'loss' if all cores were recorded as '0' (Figure 17). Cases with at least one evaluable core were included in the analysis.

The four main genes involved in DNA mismatch repair are MLH1, MSH2, MSH6 and PMS2 [388]. In a normal cell, MLH1 forms a heterodimer with PMS2 and MSH2 with MSH6. Both MLH1 and MSH2 are obligatory partners whereas PMS2 and MSH6 are secondary partners. Mutations in the MLH1 or MSH2 genes disrupt the heterodimer resulting in degradation of PMS2 and MSH6, respectively. Conversely, mutations in PMS2 and MSH6 may not result in degradation of their obligatory partners as both MLH2 and MSH2 are able to dimerise with secondary proteins such as PMS1 and MSH3 which compensate for the loss of the secondary protein [388]. In this study, MMR status was considered evaluable if at least PMS 2 and MSH 6 immunostains were evaluable. MMR was considered 'not intact' if the following IHC patterns were recorded: MLH1/PMS2 loss, MSH2/MSH6 loss, isolated MSH6 loss and isolated PMS2 loss.

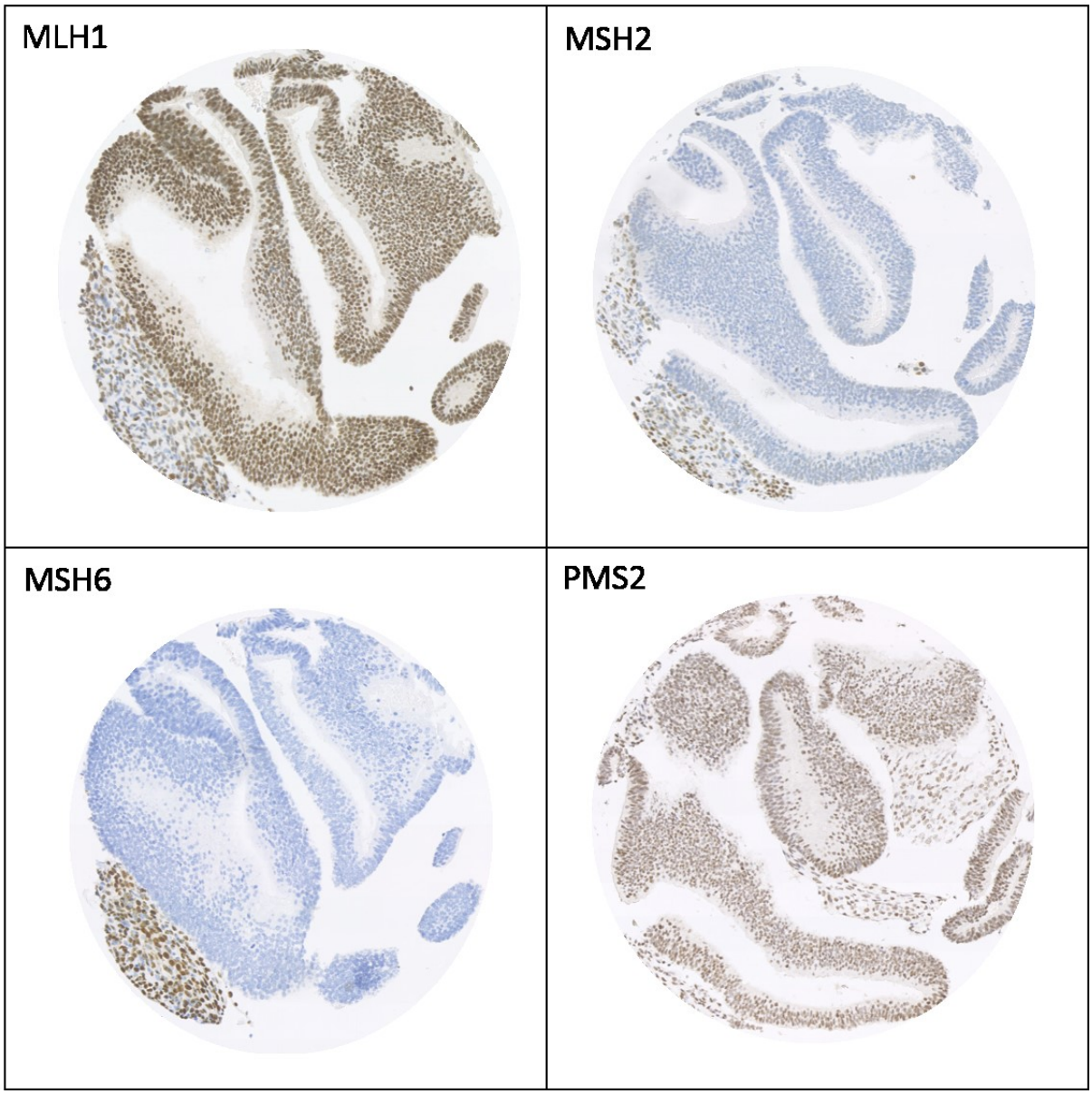

Figure 17: Example of MMR IHC staining of all four antibodies in the same tumour: Intact MLH1 and PMS2 with positive nuclear staining, loss of MSH2 and MSH6 with positive stromal cells as an internal control.

2.5.1.3 Beta-catenin

Beta–catenin staining was performed using a 1:100 dilution of the monoclonal mouse anti-human beta-catenin antibody M353901-2 (Agilent, clone b-catenin-, ERS2- 20 minutes). Normal tonsil tissue was used as the control.

Beta-catenin stain interpretation

IHC can provide a surrogate measure of *CTNNB1* mutations and has been shown to have 93-100% specificity and 80-85% sensitivity in EnEC [293]. Normal beta-catenin staining (i.e. wild-type *CTNNB1*) is expressed as membranous staining whereas *CTNNB1* mutations are expressed as abnormal nuclear accumulation of the beta-catenin protein (Figure 18).

For each core, beta-catenin staining was recorded as '0' (i.e. no mutation) if all tumour cells exhibited membranous staining [293]. Membranous staining of stromal cells acted as an internal control. Any nuclear staining of tumour cells was recorded as '1' (i.e. mutation present). Cores which were missing, or had less than 10% tumour cells were not evaluable. Cases with at least 2 evaluable cores were analysed. Cases were recorded as having an IHC staining pattern consistent with a beta-catenin mutation if at least one core was recorded as '1'.

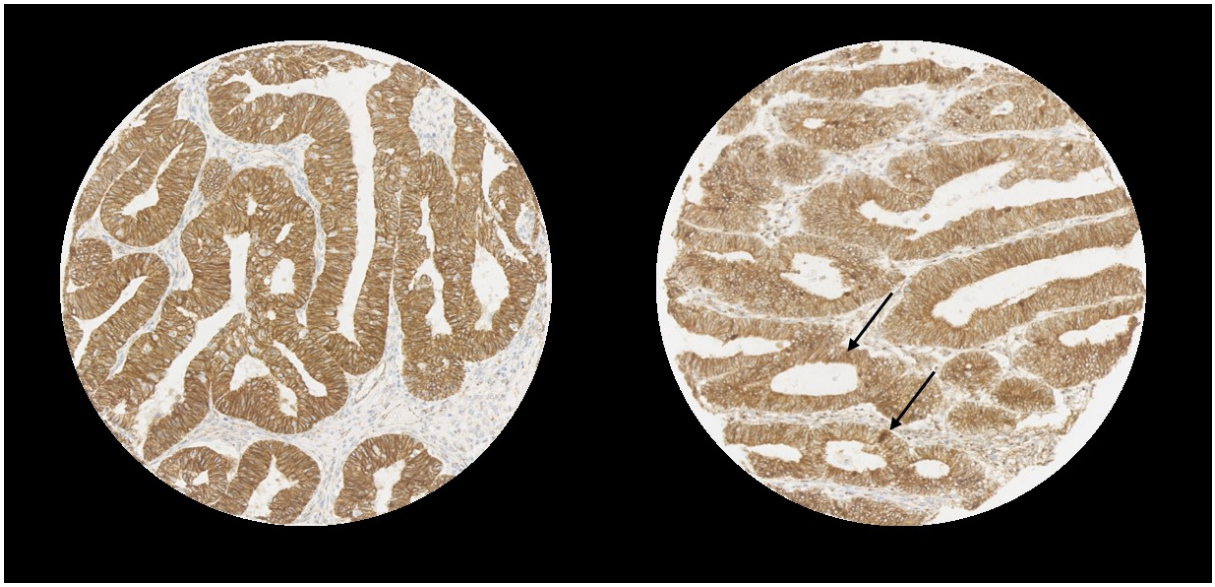

Figure 18: Example of beta-catenin IHC stain patterns: A: normal membranous staining of beta-catenin. B: nuclear staining (black arrows) of beta-catenin indicating a mutation in *CTNNB1*.

2.5.1.4 PTEN

PTEN staining was performed using a 1:100 dilution of the monoclonal mouse anti-human PTEN antibody (Sigma Aldrich, clone 6H2.1, ERS1 -20 minutes). Normal tonsil tissue was used as the control.

PTEN stain interpretation

Normal PTEN IHC expression occurs in both the nucleus and cytoplasm [389]. No 'hotspot' mutations exist. In general, loss of PTEN expression is associated with *PTEN* mutations, therefore a binary approach was adopted for this study [389, 390].

For each core, PTEN staining on tumour cells was evaluated as '1' if there was any nuclear and/or cytoplasmic staining, or '0' if there was complete loss of staining with positive stromal cells as an internal control (Figure 19). Cores which were missing or had an absence of staining of both tumour and stroma were not evaluable. Cases with at least two evaluable cores were analysed. Cases in which all cores were '1' were recorded as 'intact'. Cases in which all cores were '0' were recorded as loss. Cases with discordant cores were recorded as 'heterogeneous'.

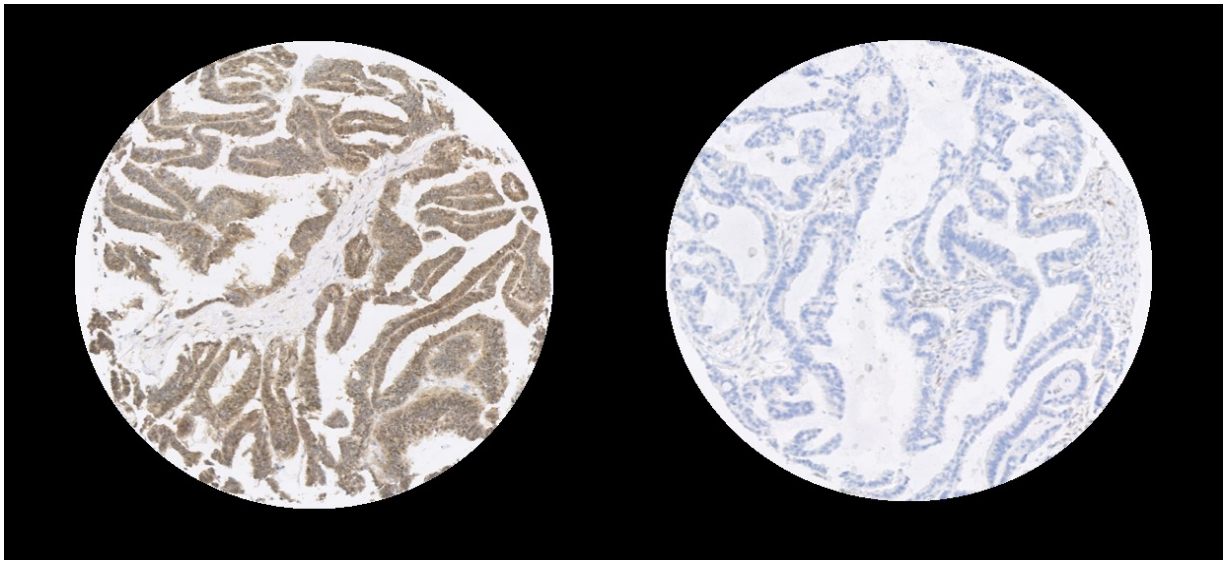

Figure 19: Example of PTEN IHC stain patterns. A: normal nuclear and membranous staining of PTEN. B: loss of nuclear PTEN staining with positive stromal cells as internal control.

2.5.1.5 ARID1A

ARID1A staining was performed using a 1:100 dilution of the polyclonal mouse anti-human ARID1A antibody HPA005456 (Sigma Aldrich, ERS1- 20minutes). Normal endometrial tissue was used as the control.

ARID1A stain interpretation

ARID1A mutations result in truncated mRNA that degrades readily which results in the loss of protein expression [315]. Loss of ARID1A on IHC has been shown to have good concordance between IHC and its mutation status [391].

For each core, ARID1A staining on tumour cells was evaluated as '1' if there was any positive nuclear stain, or '0' if there was complete loss of staining with positive stromal cell as an internal control (Figure 20). Core which were missing or had an absence of staining of both tumour and stroma were not evaluable. Cases with at least one evaluable core were evaluable. Cases with at least one core with '1' were recorded as 'intact'. Case in which all cores were '0' were recorded as loss.

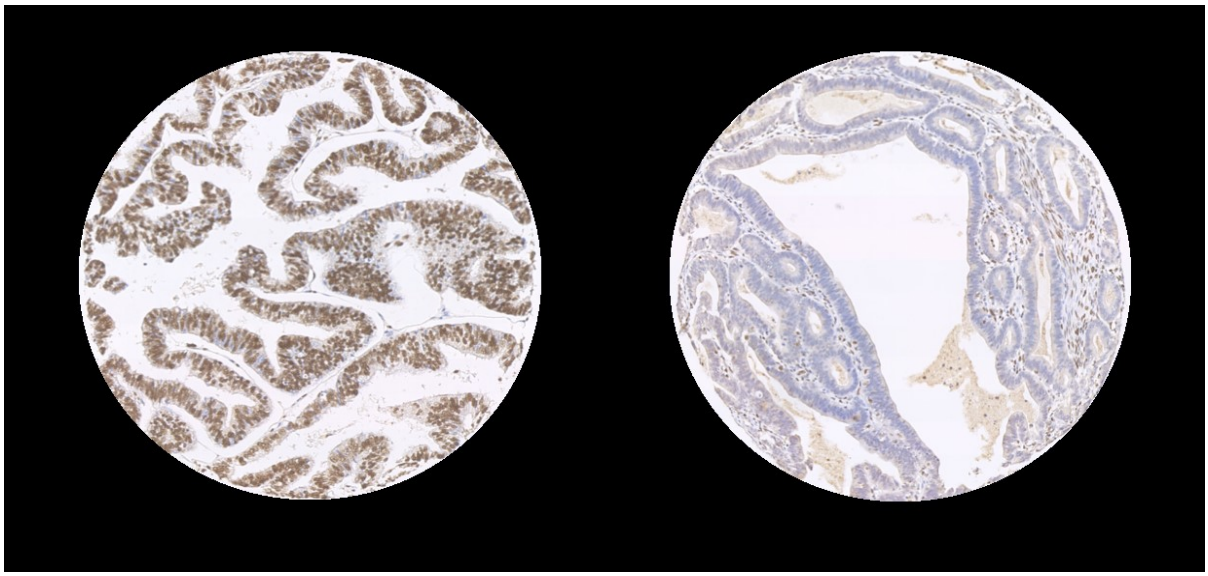

Figure 20: Example of ARID1A IHC stain patterns. A: normal nuclear staining. B: loss of nuclear staining with positive stromal cells as internal control.

2.5.1.6 PIK3CA

Staining for the PIK3CA antibody was not carried out due to discontinuation of the validated antibody (Sigma Aldrich HPA009985).

2.6 Tumour FFPE macro-dissection and DNA extraction

In section 2.2, the primary tumour cohort was assigned three IHC defined groups. The rationale for these groups were based on the following hypotheses:

Group 1: The WT1 negative p53 wild-type IHC expression low grade EnOC tumours (n=23) were pathologically homogenous and as such were hypothesised to be so at the molecular level.

Group 2 and 3: The WT1 negative p53 mutated expression IHC group which contained an admix of low grade EnOC and high grade carcinomas (n=28), and the WT1 negative p53 wild-type expression high grade carcinoma group (n=12) are likely to contain a mixture of molecular subtypes of EnOC including a subset of true high grade EnOC that could be defined genomically through whole exome sequencing (WES).

As such, DNA extraction and whole exome sequencing on all of group 2 and 3, and a randomly selected cohort of group 1 was performed. During the pathology review process, tumours which were WT1 positive but were histologically grade 1 or 2 (low grade) EnOC (n=6) were also identified. This group formed a distinct cohort of interest to the WT1 negative group and also underwent DNA extraction and subsequent WES.

A total of 69 tumours underwent macro-dissection. Ten 10μm FFPE sections were obtained from the original selected tumour block. Each H&E slide was scanned onto the Nanozoom image viewer and the area of tumour was digitally marked out and verified by CSH. Tumour cellularity was also recorded. A minimum of four 10μm FFPE sections per sample were manually macro-dissected to enrich for tumour over stroma using the marked H&E slide as a guide. Sections with a low proportion of tumour had up to eight slides macro-dissected.

DNA extraction was carried out using the QIAamp DNA FFPE Tissue Kit and Deparaffinization Solution according to the manufacturer's instructions. DNA samples extracted from FFPE tissue were quantified using the Qubit 2.0 Fluorimeter (Thermo Fisher Scientific Inc) and the Qubit DNA BR assay kit. An aliquot of each sample was then sent for WES (Appendix A). Two WT1 negative p53 mutant IHC expression tumours failed DNA extraction and were thus excluded, leaving 61 samples in the main study cohort (WT1 negative tumours) (Figure 8).

2.7 Whole exome sequencing

Whole Exome Sequencing was performed by the Wellcome Trust Clinical Research Facility in Edinburgh. Exome capture was performed using the Illumina TruSeq Exome Library Prep kit. Libraries were prepared from each DNA sample using the Illumina TruSeq Exome Library Prep kit (#FC-150-1002 - Illumina) according to the provided protocol using modifications for working with FFPE sourced material.

200ng of DNA was end-repaired to remove 3' and 5' overhangs, and fragment length was optimised using sample purification beads. A single 'A' nucleotide was added to the 3' ends of the blunt fragments to prevent them from ligating to another during the subsequent adapter ligation reaction, and a corresponding single 'T' nucleotide on the 3' end of the adapter provided a complementary overhang for ligating the adapter to the fragment. Multiple indexing adapters were then ligated to the ends of the ds cDNA to prepare them for hybridisation onto a flow cell, before 12 cycles of PCR were used to selectively enrich those DNA fragments that had adapter molecules on both ends and amplify the amount of DNA in the library suitable for sequencing. Libraries were quantified using the Qubit 2.0 Fluorometer and the Qubit DNA HS assay (#Q32854 - ThermoFisher) and the size distribution of fragments was assessed using the Agilent Bioanalyser with the DNA HS Kit (#5067-4626 - Agilent).

DNA libraries containing unique indexes were combined in pools of six, and then target regions of the DNA were bound with capture probes. Streptavidin Magnetic Beads were then used to capture probes hybridised to the targeted regions of interest and a series of washes removed nonspecific binding from the beads. This process was repeated to ensure high specificity of the captured regions. Captured enriched library was then purified before eight cycles of PCR amplification and a final purification step to remove unwanted products.

Exome-captured sequencing library pools were quantified using the Qubit 2.0 Fluorometer and the Qubit DNA HS assay (#Q32854 - ThermoFisher) and the size distribution of fragments was assessed using the Agilent Bioanalyser with the DNA HS Kit (#5067-4626 - Agilent). Fragment size and quantity measurements were used to calculate molarity for each library pool.

WES was performed using the NextSeq 500/550 High-Output v2 (150 cycle) Kit (# FC-404-2002) on the Illumina NextSeq 550 (Illumina, Inc., San Diego, CA, USA), achieving a median per-sample mean target coverage of 148X (range 50X-327X). Three samples achieved <60M reads. Data was aligned to the human reference genome using bwa bwa-0.7.17 [392], duplicates marked and base quality scores recalibrated with the GenomeAnalysisToolkit (GATK) v4 [393] in the bcbio 1.0.6 pipeline (https://github.com/bcbio/bcbio-nextgen).

2.8 Variant calling and classification

The bioinformatics analysis pipeline was performed and analysed by Dr John Thompson, Dr Robert Hollis and the MRC Human Genetics Unit, University of Edinburgh. Variant calling was performed using a majority vote from three variant caller algorithms; VarDict [394], Mutect2 [395], Freebayes [396]. Filtering for C>T (FFPE artifacts) and G>T (oxidation artifacts) was applied using GATK (CollectSequencingArtifactMetrics and FilterByOrientationBias). Resulting VCF files were then analysed in R using the maftools package (https://bioconductor.org/packages/release/bioc/html/maftools.html.

Datasets were filtered for common population variants using the 1000 genomes (1000 genomes phase 1 snp and indel dataset; http://www.internationalgenome.org/) and the Exome Aggregation consortium (ExAC) reference datasets(ExAC.0.3.GRCh38 : http://exac.broadinstitute.org/). Using the Polymorphism Phenotyping (PolyPhen) [397] and/or Sorting Intolerant from Tolerant (SIFT) [398] prediction tools as well as the NCBI clinVar database (https://www.ncbi.nlm.nih.gov/clinvar/) which aggregates pathogenicity reports associated with genomic variants, variants predicted not to affect protein function were discarded as likely non-functional events. Filtering was then applied to define high impact mutations where the variant allele frequency of a given mutation was > 10% across regions with a minimum read coverage of 20X.

2.9 Analysis of mutational status

Mutational and copy number analysis in sections 2.9, 2.10 and 2.11 was performed by Dr John Thompson. Supervised mutational analysis of the 61 WT1 negative samples was carried out by investigating the mutational landscape across 75 commonly mutated genes previously reported in either endometrial, ovarian or pan cancer studies (Appendix B) [131, 356]. This list of genes were curated by Dr John Thompson. The mutational status of these genes was visualised using the R package maftools. The same analysis was applied to the six WT1 positive low grade EnOC identified during the pathology review process.

2.10 Unsupervised clustering analysis

Unsupervised clustering analysis was performed across the top 100 differentially mutated genes within the dataset. Samples were clustered by Euclidian and Ward methods based on the overall Pearson correlation score of these binary signatures. Heat maps were drawn in R using the ggplot package [399].

2.11 Copy number variation (CNV) detection

Copy number analysis was performed by using GeneCN pipelines using Bio-DB-HTS version 2.10 (https://github.com/wwcrc/geneCN). Copy number (CN) analysis for the 75 gene panel described above was performed using GeneCN pipelines using Bio-DB-HTS version 2.10 (https://github.com/wwcrc/geneCN) to identify significant CN gain or loss (CN score >3 standard deviations from reference, $P<0.05$) using the pooled WT1 negative *TP53* wild-type (*TP53*^{wt}) samples as a reference. Heat maps of CN gain and loss were drawn in R using the “heatmap2” function in the “gplots” package.

2.12 Statistical Analysis

All statistical analyses were performed using R version 3.5.1 by Dr Robert Hollis. Comparisons of continuous data were made with the Mann Whitney-U test or T-test, as appropriate. Survival analysis was performed using the Survival package in R using cox proportional hazards regression analysis [400]. Owing to the established concordance between *TP53* mutations and p53 IHC [384], *TP53* mutation status was inferred as wild-type in p53 IHC wild-type samples to supplement multivariable analysis of this marker in the WES cohort (referred to as *TP53* mutation status in the multivariable analysis). Multivariable analyses accounted for disease stage, patient age at diagnosis, decade of patient diagnosis and extent of residual disease following surgical cytoreduction. Comparisons of frequency as well as mutation status with IHC were performed using the Chi-squared test or Fisher’s exact test, as appropriate.

3. Clinical characteristics of EnOC

3.1 Introduction

EnOC usually present as early stage, low grade disease and are associated with endometrioisis [209, 220] and synchronous EnEC [129, 231]. Low grade EnOC are morphologically distinct with a classical IHC profile which is WT1 negative, p53 wild type expression, and strong ER expression [129, 226]. Due to refinement in pathological definitions over time, it is now recognised that many high grade EnOC are in fact misclassified HGSOC, a finding that is supported by several transcriptomic studies [243, 246-248]. True high grade EnOC are increasingly rare and are challenging to diagnose. They share morphological overlap with both HGSOC, as well as the rare and aggressive de-differentiated carcinomas which are a mix of low grade EnOC and undifferentiated carcinoma [257]. As discussed in chapter 1, WT1 IHC is a useful and validated marker which can assist in discriminating between these sub-types (high grade EnOC; WT1 negative, HGSOC; WT1 positive), and can help reduce inter-observer diagnostic variation [224, 229, 230, 243].

In general, EnOC is regarded as having a better prognosis compared to HGSOC [226, 229, 272, 274], however it is less certain whether this is mostly due to the majority of EnOC presenting at an earlier stage. Moreover, most studies did not perform stage for stage comparison [274, 276-278] or contemporary pathology review according to WHO 2014 criteria on their cohorts [272, 279]. As such, these studies are likely to have included a proportion of misclassified HGSOC which would have influenced clinical outcomes**.** In those studies that did perform contemporary pathology review, most did not utilise IHC to confirm the diagnosis of high grade EnOC with a reliance on histology for pathological diagnosis [224, 226, 282]. This once again raises doubt as to the clinical outcomes reported in those studies as differentiation between HGSOC and high grade EnOC is a notorious area of poor reproducibility based on histology alone [229]. The clinical behaviour of high grade EnOC compared to HGSOC in terms of treatment responses and clinical outcomes have also been under-investigated [224].

The management of EnOC largely mirrors that of HGSOC. In Europe, the standard of care for surgery includes total abdominal hysterectomy, bilateral salpingo-oophorectomy, infracolic omentectomy, and lymph node sampling [2]. Systematic aortic and pelvic lymphadenectomy is however controversial [17]. In early stage EnOC, the rates of lymph node involvement are reported to be approximately 2% [284-286], with some studies reporting higher rates for high grade EnOC [234]. Some retrospective studies performed have not found a survival benefit with lymphadenectomy [284-286], whereas other studies of historically diagnosed EnOC have found lymphadenectomy to be an independent factor of prognosis [286-289]. However, it is plausible that the presence of undiagnosed HGSOC in these cohorts may have influenced these results.

As discussed in chapter 1.5.7.2, the survival benefit of adjuvant chemotherapy in early stage EnOC is difficult to quantify as the early adjuvant trials performed included all histological subtypes of EOC [27]. Retrospective studies have been performed to identify prognostic factors in order to define a cohort of patients with EnOC who can be spared the toxicity of adjuvant chemotherapy. These have included grade [291], sub-staging of IC disease [274], ER and PR receptor status [255],β-catenin and CDX2 [293], and L1CAM [275]. However, these biomarkers were not investigated as a multi-marker panel in a comprehensive multivariable analysis. As such, it remains standard of care to consider adjuvant chemotherapy for all patients with stage 1C and above EnOC of any grade, and stage 1B grade 3 EnOC [41]. The management of advanced stage and relapsed EnOC is in line with that of HGSOC with platinum based chemotherapy forming the main stay of treatment. Maintenance PARP inhibitors are used for platinum sensitive relapsed high grade EnOC following a response to platinum based chemotherapy [149] or as first line maintenance treatment in germline *BRCA* mutated high grade EnOC [294]. As these tumours mostly display strong ER expression, endocrine therapy is often used as the preferred option at first relapse. The platinum sensitivity of EnOC is however not well understood and has been largely extrapolated from that of HGSOC.

As described in chapter 2, pathology review performed in this study identified 125 WT1 negative carcinomas stratified by p53 IHC and grade. These three IHC defined cohorts were classical low grade EnOC (WT1 negative, p53 wild-type expression), low grade EnOC and high grade carcinomas with WT1 negative p53 aberrant IHC expression, and WT1 negative p53 wild-type expression high grade carcinomas. The WT1 negative high grade carcinomas included for review had high grade serous, high grade endometrioid and undifferentiated histology due to the well described morphological overlap between these tumours.

In this chapter, it is hypothesised that the defined WT1 negative cohort in this study display favourable prognosis and platinum sensitivity lower than that reported in studies of platinum naïve or sensitive relapsed HGSOC. Although one of the objectives of this study was to clinically compare low grade EnOC with high grade EnOC, the pathological definitions used to define high grade disease included tumours with serous and undifferentiated morphology. Furthermore, there were insufficient numbers of tumours with histologically defined high grade EnOC. As such, this clinical comparison was not performed in this chapter. Instead, this chapter is purely descriptive, and presents the clinical characteristics, survival and platinum sensitivity of the WT1 negative cohort. It was also hypothesised that the presence of high grade carcinomas and that of low grade EnOC with aberrant p53 expression on IHC would impact on survival outcomes, as such the same clinical characteristics were reported separately for the classical low grade EnOC cohort.

3.2 Clinical characteristics

WT1 negative cohort:

In the WT1 negative cohort, 22 (17.6%), 48 (38.4%), 33 (26.4%), and 22 (17.6%) were diagnosed in the 1980s, 1990s, 2000s and 2010s, respectively. The median age of diagnosis was 58 years (range 28 years to 88 years). Median body mass index of patients was 25.3 (range 18-44). Median size of the ovarian mass was 13.0cm (range 1.4cm to 30.0cm) of which 17.5% were bilateral. 20 (16.0%) had synchronous endometrial cancers, and 42 (33.6%) had endometrioisis. The majority of patients presented with stage I (40.0%) and stage II (37.6%) disease. Of these, the majority of stage I patients had IC disease (28 of 50; 56.0%) and stage II patients had IIC disease (30 of 47; 63.8%). Notably, 7 (77.8%) stage IV patients had visceral metastases at presentation with liver metastases as the most common site (Figure 21).

102 (81.6%) patients were surgically cyto-reduced to less than 2cm, of which 65 (52.0%) were recorded as 0 cm. 5 (4.0%) had unknown residual disease.

Of the 97 patients with early stage (stage I and II disease), 74 (76.3%) underwent full surgical staging (defined as total abdominal hysterectomy, bilateral salpingo-oopherectomy, omental biopsy or omentectomy, and washings). Two patients underwent lymph node sampling whilst no patients underwent lymphadenectomy. One patient with stage IA disease underwent adjuvant chemotherapy due to the absence of lymph node sampling. 18 (18.6%) were under-staged, with the majority (12; 66.7%) due to the omission of an omentectomy.

Classical low grade EnOC:

87 tumours were diagnosed as classical low grade EnOC (87.4% G1, 12.6% G2). Clinical characteristics are summarised in Table 6. The majority of patients (75; 86.2%) presented with early stage disease with only 2.3% as stage IV. Most patients were surgically cyto-reduced to less than 2cm (58.6% 0cm, 31.0% <2cm). 13 (17.3%) patients with early stage disease were under-staged.

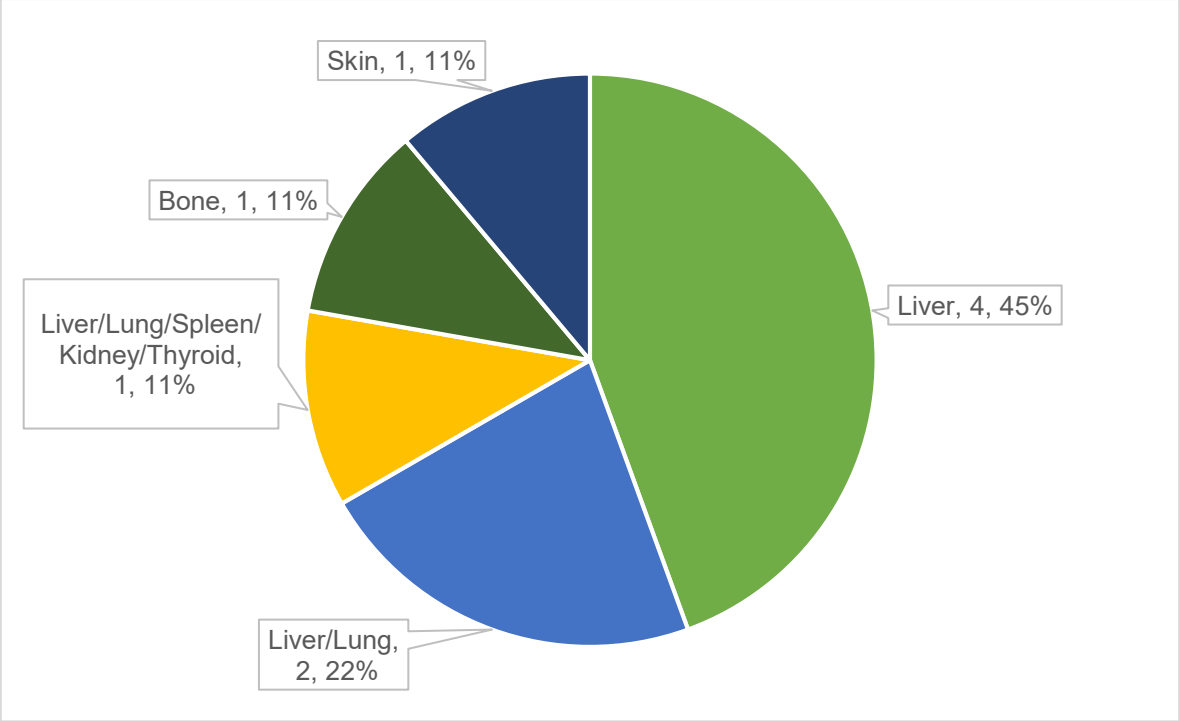

Figure 21: Site of metastases in Stage IV disease.

Table 6: Clinical characteristics of the WT1 negative cohort (A) and classical low grade EnOC (B).					
		A: (n=125)		B: (n=87)	
Variables		**n**	**%**	**n**	**%**
Age		58(28-88)	-	57 (28-88)	-
BMI (A: n=68; B: n=44)		25.3 (range 18-44)	-	25(18-44)	-
Endometrial cancer		20	16.0	17	19.5
Endometrioisis		42	33.6	34	39.1
Histology	G1 EnOC	80	64.0	76	87.4
	G2 EnOC	19	15.2	11	12.6
	G3 EnOC	8	6.4	0	0.0
	HGS	15	12.0	0	0.0
	Undifferentiated	3	2.4	0	0.0
Year of Diagnosis	1980s	22	17.6	17	19.5
	1990s	48	38.4	30	34.5
	2000s	33	26.4	26	29.9
	2010s	22	17.6	14	16.1
Stage I	all	50	40.0	38	43.7
	IA	20	16.0	14	16.1
	IB	2	1.6	1	1.1
	IC	28	22.4	23	26.4
Stage II	all	47	37.6	37	42.5
	IIA	4	3.2	3	3.4
	IIB	11	8.8	9	10.3
	IIC	30	24.0	24	27.6
	UK sub-staging	2	1.6	1	1.1
Stage III	all	17	13.6	8	9.2
	IIIA	2	1.6	1	1.1
	IIIB	2	1.6	1	1.1
	IIIC	9	7.2	4	4.6
	UK sub-staging	4	3.2	2	2.3
Stage IV	all	9	7.2	2	2.3
	IVA	0	0.0	0	0.0
	IVB	9	7.2	2	2.3
UK stage		2	1.6	2	2.3
Residual disease[a]	<2cm[b]	102	81.6	78	89.7
	≥ 2cm[C]	18	14.4	6	6.9
	UK	5	4.0	3	3.4
Surgical staging (I+II)	Fully staged	74	76.3	57	76.0
(A: n=97; B: n=75)	Under staged	18	18.6	13	17.3
	UK	5	5.2	5	6.7

Legend: G=grade; EnOC=endometrioid ovarian carcinoma; HGS=high grade serous morphology; BMI=body mass index; UK=unknown.[a]Classification of optimal surgical cytoreduction changed over time; some of the patients were diagnosed at a time when resection to <2cm was considered optimal. [b]65 (52%) patients were cyto-reduced to 0cm in the whole cohort. [C] 2 patients were inoperable.

3.2.1 Therapy received (WT1 negative cohort)

67 (53.6%), 14 (11.2%), 10 (8.0%) received one, two and three lines of chemotherapy, respectively. 33 (26.4%) received no chemotherapy. A total of 128 courses of chemotherapy were delivered, of which 102 (79.7%) were platinum based. 84(82.4%) were delivered in the platinum naïve setting, 15 (14.7%) in the platinum sensitive setting, 1 (1.0%) in the partially platinum sensitive setting and 2 (2.0%) in the platinum resistant setting (Table 7).

In early stage disease (n=97), 66 (68.0%) patients received adjuvant chemotherapy, of which 57 (86.4%) were platinum-based (Table 7). The median number of cycles received of adjuvant platinum chemotherapy was six (range 3-6), and 44 (77.2%) received all six cycles. In advanced stage disease (n=26), 19 (73.1%) patients received platinum based chemotherapy as their first line of treatment. The median number of platinum cycles received was six (range1-6), and 63.2% received all 6 cycles.

Eight (6.4%) patients, of which five were classical low grade EnOC, underwent an attempt at secondary cytoreductive surgery as second line treatment. The median time to relapse was 2.8 years (1.4-6.1 years). All 8 patients recurred as solitary masses (six pelvic, two nodal). Six (75.0%) achieved complete macroscopic cytoreduction. Of these, only two patients died from ovarian cancer after a median follow up time of 11.5 years (4.0-24.2 years). 11 (8.8%) patients received endocrine therapy of which eight were classical low grade EnOC (detailed in chapter 4).

Table 7: Treatment received by the WT1 negative cohort (A) and classical low grade EnOC (B).					
		A: (n=125)		B: (n=87)	
Variables		**n**	**%**	**n**	**%**
Chemotherapy	none	33	26.4	26	29.9
	1 line	67	53.6	48	55.2
	2 lines	14	11.2	10	11.5
	3 or more lines	10	8.0	2	2.3
	UK	1	0.8	1	1.1
No. of chemotherapy courses received	Platinum	102	79.7	65	86.7
(A:n=128; B:n=75)	non-platinum	26	20.3	10	13.3
Line of treatment platinum received in	1	80	78.4	54	83.1
(A:n=102; B:n=65)	2	17	16.7	9	13.8
	3	3	2.9	2	3.1
	4	2	2.0	1	1.5
Platinum setting	Naïve	84	82.4	56	86.2
(A:n=102; B:n=65)	Platinum sensitive	15	14.7	9	13.8
	Partially platinum sensitive	1	1.0	0	0.0
	Platinum resistant	2	2.0	0	0.0
Secondary cytoreduction	Inoperable	2	25.0	2	40.0
(A:n=8; B:n=5)	Completely cyto-reduced	6	75.0	3	60.0
Endocrine therapy	none	112	89.6	78	89.7
	1 line	9	7.2	7	8.0
	2 lines	2	1.6	1	1.1
	UK	2	1.6	1	1.1
Adjuvant treatment stage I/II	platinum only	33	34.0	23	30.7
(A:n=97; B:n=75)	platinum-taxane	21	21.6	17	22.7
	platinum-other	3	3.1	3	4.0
	chlorambucil	9	9.3	5	6.7
	None	30	30.9	27	36.0
	UK	1	1.0	1	1.3
Adjuvant platinum stage I/II	Median no. of cycles	6 (3-6)	NA	6(4-6)	NA
(A:n=57; B:n=43)	6 cycles received	44	77.2	33	76.7
First treatment Stage III/IV	platinum only	13	50.0	5	50.0
(A:n=26; B:n=10)	platinum-taxane	2	7.7	1	10.0
	platinum-other	4	15.4	2	20.0
	endocrine therapy	1	3.8	1	10.0
	None	6	23.1	1	10.0
platinum chemotherapy (A;n=19; B:n=8)	Median no. of cycles	6(1-6)	NA	6(4-6)	NA
	6 cycles received	12	63.2	6	75.0

3.2.2 Overall Survival

The median duration of follow up was 7.5 years (range 0.1-29.7 years). 61 (48.8%) deaths occurred, of which 38 (30.4%) were ovarian cancer related with the remaining 23 (18.4%) due to other causes. The overall median DSS and RFS was not reached at ten years. For the WT1 negative cohort, overall five year RFS and DSS was 69.1% and 73.2%, and ten year RFS and DSS was 63.6% and 67.0%, respectively (Table 8, Figure 22). For the classical low grade EnOC cohort, overall five and ten year RFS was 83.0% and 76.7%, and DSS was 86.4% and 79.7%, respectively (Table 9, Figure 23).

3.2.3 Survival by stage

For the WT1 negative cohort, five and ten year DSS was 93.7% and 87.6% for stage I, 81.6% and 71.1% for stage II, 29.4% for both in stage III and 0% for both in stage IV (Table 8, Figure 24). Median DSS for stage III and IV disease was 2.9 years and 0.7 years, respectively. In the classical low grade EnOC cohort, five and ten year DSS was 94.3% and 90.5% for stage I, and 88.5% and 75.4% for stage II disease, respectively. Five and ten year DSS for advanced stage disease (n=10) was 50.0% with a median of 7.8 years (Table 9, Figure 25). Similar patterns were observed for five and ten year RFS in both cohorts. Median RFS and DFS was not reached for early stage disease.

3.2.4 Other clinical variables of prognosis

Upon univariable analysis, only age, grade and residual disease was significant for RFS and DSS, whereas no differences were observed for decade of diagnosis (Table 10). Multivariable analysis was then performed together with molecular variables and reported in chapter 5.

Table 8: Survival by stage in the WT1 negative cohort (n=125).

	Disease specific survival					Relapse free survival				
	Median/ years	Five year (%)	Ten year (%)	HR (95% CI)	P	Median/ years	Five year (%)	Ten year (%)	HR (95% CI)	P
Overall	N/R	73.2	67.0	-		N/R	69.1	63.6	-	
Stage I (n=50)	N/R	93.7	87.6	0.08 (0.03-0.22)	<0.001	N/R	91.8	86.4	0.08 (0.03-0.22)	<0.001
Stage II (n=47)	N/R	81.6	71.0	0.20 (0.09-0.46)	<0.001	N/R	74.7	65.6	0.23 (0.11-0.49)	<0.001
Stage III (n=17)	2.9	29.4	29.4	ref		1.5	23.5	23.5	ref	
Stage IV (n=9)	0.7	0.0	0.0	5.42 (2.02-14.54)	<0.001	0.5	0.0	0.0	5.72 (2.18-15.00)	<0.001

Legend: N/R=not reached. 2 patients had unknown stage.

Table 9: Survival by stage in classical low grade EnOC (n=87).

	Disease specific survival					Relapse free survival				
	Median/ years	Five year (%)	Ten year (%)	HR (95% CI)	P	Median/ years	Five year (%)	Ten year (%)	HR (95% CI)	P
Overall	N/R	86.4	79.7	-	-	N/R	83.0	76.7	-	-
Stage I (n=38)	N/R	94.3	90.5	0.10 (0.03-0.42)	0.001	N/R	91.8	88.3	0.12 (0.03-0.42)	<0.001
Stage II (n=37)	N/R	88.5	75.4	0.27 (0.09-0.81)	0.020	N/R	85.4	73.5	0.26 (0.09-0.76)	0.014
Stage III/IV (n=10)	7.8	50.0	50.0	ref	ref	2.0	40.0	40.0	ref	ref

Legend: NR=not reached. 2 patients had unknown stage.

Table 10: Univariable analysis of clinical prognostic variables.

Variable		Disease specific survival		Relapse free survival	
		HR (95% CI)	P	HR (95% CI)	P
Decade of diagnosis	1980s	ref		ref	
	1990s	1.03(0.47-2.29)	0.935	0.84 (0.39-1.81)	0.667
	2000s	0.63 (0.24-1.62)	0.335	0.58 (0.23-1.42)	0.233
	2010s	0.26 (0.06-1.21)	0.085	0.37 (0.12-1.19)	0.095
Residual disease[a]	<2cm	0.03 (0.01-0.07)	<0.001	0.04 (0.01-0.09)	<0.001
	2-5cm	0.3 (0.10-0.83)	0.021	0.31 (0.11-0.86)	0.025
	>5cm	ref		ref	
Age	years	1.05 (1.01-1.08)	0.006	1.03 (1.00-1.06)	0.031
Grade	1	0.19 (0.09-0.40)	<0.001	0.23 (0.12-0.46)	<0.001
	2	0.61 (0.26-1.42)	0.248	0.87 (0.40-1.93)	0.74
	3	ref		ref	

Legend: [a]Classification of optimal surgical cytoreduction changed over time; some of the patients were diagnosed at a time when resection to <2cm was considered optimal.

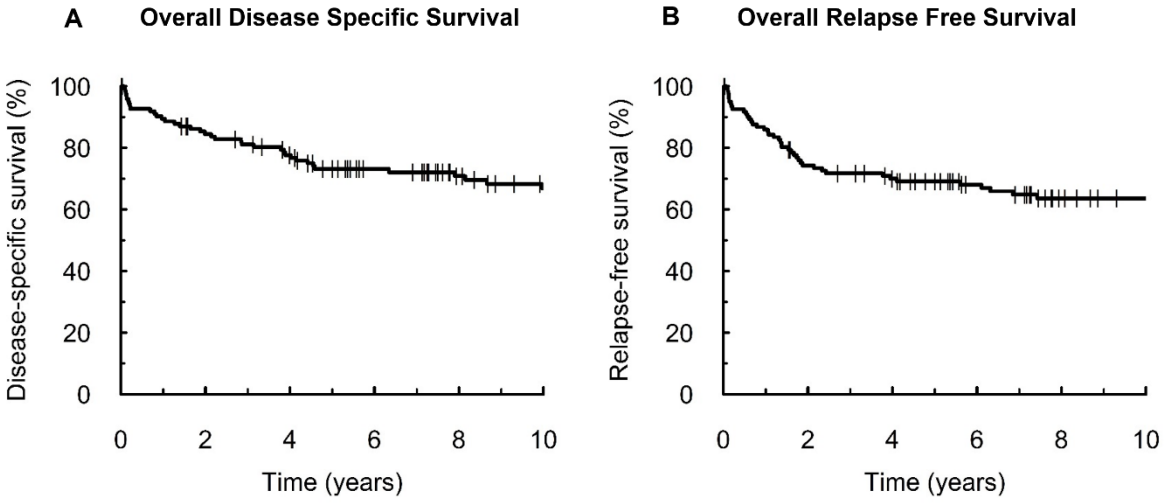

Figure 22: Overall disease specific survival (A) and relapse free survival (B) in the WT1 negative cohort.

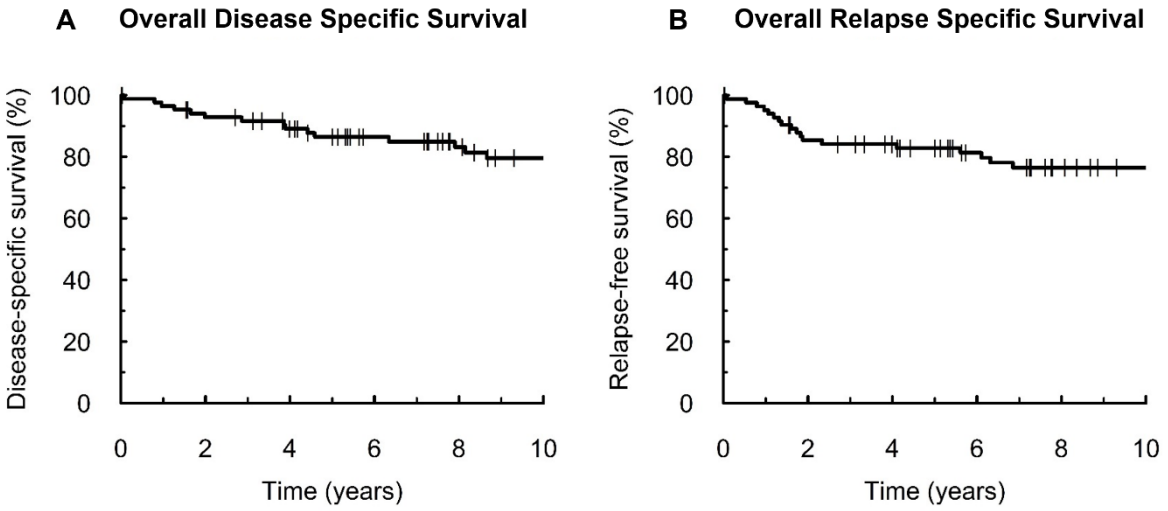

Figure 23: Overall disease specific survival (A) and relapse free survival (B) in the classical low grade EnOC cohort.

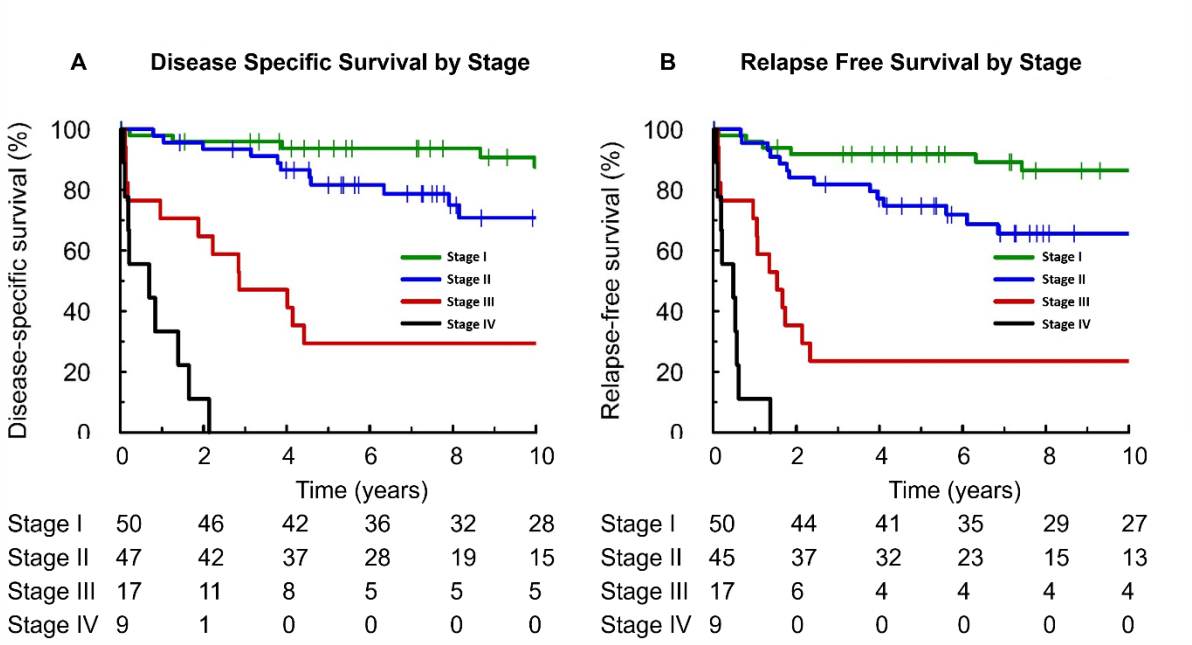

Figure 24: Disease specific survival (A) and relapse free survival (B) by stage in the WT1 negative cohort.

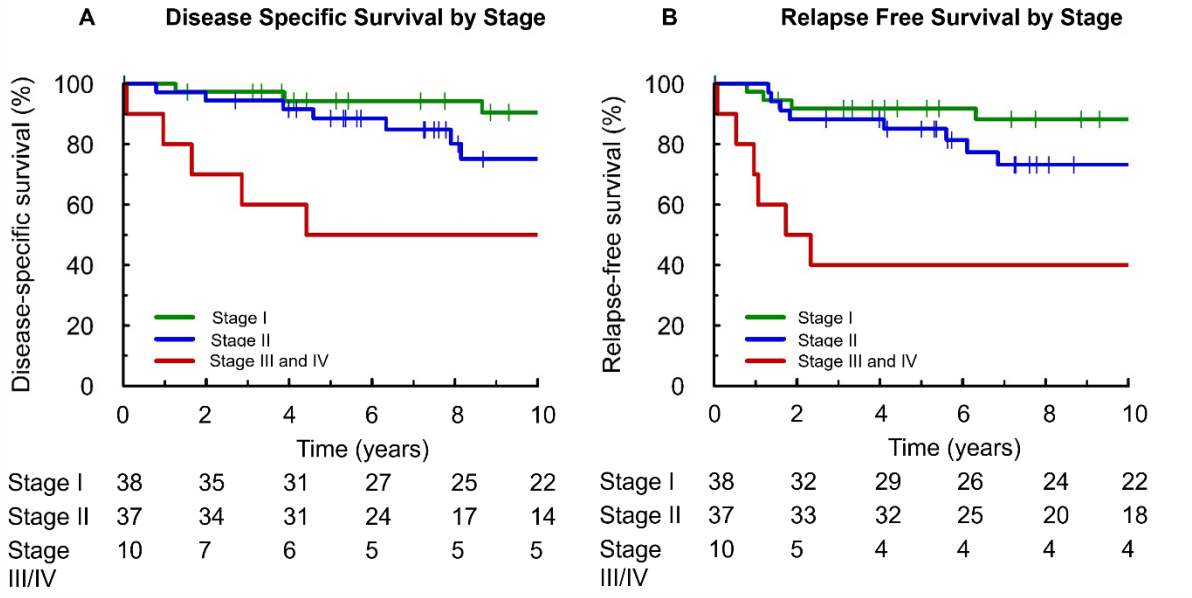

Figure 25: Disease specific survival (A) and relapse free survival (B) by stage in the classical low grade EnOC cohort. Stage III (n=8) and IV (n=2) patients combined due low numbers.

3.3 Characteristics and survival of stage I disease

22 patients were diagnosed with stage IA/IB disease (Table 11). Ten year RFS and DSS was 82.5% and 80.6%, respectively. 14(63.6%) underwent full surgical staging and 5 (22.7%) received adjuvant platinum chemotherapy. The three patients who died of their disease had G1 EnOC, G3 EnOC and undifferentiated carcinoma, and recurred at 6.3 years, 7.4 years and 0.1 years, respectively.

Of the 28 patients diagnosed with stage IC disease, 71.4% underwent full surgical staging. Overall ten year RFS and DSS was 89.3% and 92.5%, respectively. No differences in RFS (P=0.67) or DSS (P=0.89) were observed between those who received adjuvant chemotherapy versus those who did (Table 12).

Table 11: Characteristics of stage IA/IB disease (n=22).

		N	%
Stage	IA	20	90.9
	IB	2	9.1
Ten year	RFS	NA	82.5
	DSS	NA	80.6
Histology	G1 EnOC	14	63.6
	G2 EnOC	3	13.6
	G3 EnOC	2	9.1
	HGS	1	4.5
	Undiff	1	4.5
Full surgical staging	Yes	14	63.6
	No	6	27.3
	UK	2	9.1
Platinum chemotherapy	Yes	5	22.7
	No	17	77.3

Legend: RFS=relapse free survival; DSS=disease specific survival; EnOC=endometrioid histology; HGS=high grade serous morphology; Undiff=undifferentiated histology;UK=unknown.

Table 12: Characteristics of stage IC disease (n=28).

		Adjuvant Platinum (n=13)		No chemotherapy/ non-platinum (n=15)			Overall	
		N	%	N	%	P-value	N	%
ten year	RFS	-	92.3	-	86.7	0.67	-	89.3
	DSS	-	92.3	-	92.9	0.89	-	92.5
Histology	G1 EnOC	10	76.9	12	80.0	NC	22	78.6
	G2 EnOC	0	0.0	3	20.0	NC	3	10.7
	G3 EnOC	3	23.1	0	0.0	NC	3	10.7
Full surgical staging	Yes	8	61.5	12	80.0	NC	20	71.4
	No	4	30.8	3	20.0	NC	7	25.0
	UK	1	7.7	0	0.0	NC	1	3.6

Legend: RFS=relapse free survival; DSS=disease specific survival; EnOC=endometrioid histology; NA=not applicable; NC=not calculated due to low numbers; UK=unknown.

3.4 Patterns of relapse in early stage disease

Of 97 patients with early stage disease, 22 (22.7%) relapsed. 20 patients had radiological or intraoperative evidence of disease recurrence (Table 13). Of these, ten (45.5%) recurred as a solitary pelvic mass, with lower frequencies in other sites (13.6% nodal, 13.6% visceral, 2% peritoneal, 9.1% other). All ten cases which recurred as a solitary pelvic mass had low grade EnOC (six G1, four G2) at diagnosis. 13 (59.1%) recurrences were confirmed pathologically as recurrent ovarian carcinoma. Pathology review of recurrent samples was not performed in this study and details of the post biopsy pathology report are detailed in Table 14. Of the four samples which had grade of the tumour recurrence reported, three were higher grade than the initial diagnosis of grade 1 EnOC (one high grade EnOC, one mixed grade 3 serous/ EnOC, one grade 2 EnOC).

Table 13: Patterns of relapse in early stage disease (n=22).			
		N	**%**
Method of detection	CT	14	63.6
	US	3	13.6
	Laparotomy	2	9.1
	MRI	1	4.5
	CA125 only relapse	1	4.5
	UK	1	4.5
Site	Nodal only	3	13.6
	Visceral[a]	3	13.6
	Solitary pelvic mass	10	45.5
	Peritoneal	2	9.1
	Other[b]	2	9.1
	NA	1	4.5
	UK	1	4.5
Relapse	Biopsy confirmed	13	59.1
	Not biopsied	7	31.8
	No disease[c]	1	4.5
	UK	1	4.5

[a]Lung or liver metastases and peritoneal/pelvic disease.
[b]1 patient had pelvic mass and paraaortic lymphadenopathy,1 patient had chest/abdominal wall metastases and paraaortic lymphadenopathy
[c] no radiologically detectable disease (CA125 only relapse)
Legend: UK=unknown.

Table 14: Pathology of recurrent disease (n=13).	
Pathology at diagnosis	**Pathology at relapse[a]**
G1 EnOC	Consistent with ovarian cancer
G1 EnOC	Consistent with ovarian cancer
G1 EnOC	Endometrioid no grade
G1 EnOC	Endometrioid no grade
G1 EnOC	G2 EnOC
G1 EnOC	G3 EnOC
G1 EnOC	Mixed grade 3 serous/endometrioid
G2 EnOC	G1 EnOC
G2 EnOC	Consistent with ovarian cancer
G2 EnOC	Endometrioid no grade
G3 EnOC	Papillary serous
HGS	Consistent with ovarian cancer

[a] As per the pathology report.
Legend: G=grade, EnOC= WT1 negative endometrioid ovarian carcinoma; HGS= high grade serous morphology

3.5 Platinum responses

99 patients received platinum chemotherapy in the platinum naïve or platinum sensitive (≥12 month from last platinum) setting. Of these, 18 (18.2%) and 13 (13.1%) patients were evaluable for radiological and CA125 responses, respectively, in the WT1 negative cohort (Table 15). Most patients received single agent platinum only (83.3% of radiology evaluable cohort, 84.6% CA125 evaluable cohort). Overall radiological response rate was 44.5% (5.6% CR, 38.9% PR), 11.1% SD, and 44.4% PD. Overall CA125 response rate was 69.3 %(38.5% CR, 30.8% PR), 23.1% SD and 7.7% PD. The median number of CA125s recorded was 7 (range 3-10).

65 classical low grade EnOC patients received platinum-based chemotherapy in the platinum naïve and platinum sensitive setting. Of 10 evaluable radiological responses, 50.0% demonstrated PR, 20.0% SD and 30.0% PD. All five evaluable CA125 responses demonstrated response or stabilisation of CA125 (20.0% CR, 40.0% PR, 40.0% SD).

Table 15: Radiological and CA125 response to platinum chemotherapy.

Variable		WT1 negative cohort				Classical low grade EnOC			
		Radiology (n=18)		CA125 (n=13)		Radiology (n=10)		CA125 (n=5)	
		n	%	n	%	n	%	n	%
Setting	Platinum Naïve	9	50	6	46.2	5	50.0	2	40.0
	Platinum sensitive	9	50	7	53.8	5	50.0	3	60.0
Chemo	Platinum only	15	83.3	11	84.6	10	100.0	5	100.0
	Platinum/taxane	2	11.1	1	7.7	0	0.0	0	0.0
	Platinum/other	1	5.6	1	7.7	0	0.0	0	0.0
Response	CR	1	5.6	5	38.5	0	0.0	1	20.0
	PR	7	38.9	4	30.8	5	50.0	2	40.0
	SD	2	11.1	3	23.1	2	20.0	2	40.0
	PD	8	44.4	1	7.7	3	30.0	0	0.0

Legend: EnOC=endometrioid ovarian carcinoma; CR=complete response; PR=partial response; SD=stable disease; PD=progressive disease.

3.5.1 Stage IV disease

Due to the poor survival demonstrated in stage IV disease, the clinical characteristics and response to platinum chemotherapy specifically in this cohort were investigated. Six (66.7%) of nine stage IV patients received first line platinum chemotherapy. Three were G2 EnOC, and three were high grade carcinomas. Of these, five (83.3%) patients were platinum refractory, and one patient had radiological PR. Of these five patients, two died rapidly after one cycle (37 days and 69 days), three experienced disease progression after at least five cycles. All three evaluable patients had a CA125 response (two CR and one PR). The median DSS in those that received platinum chemotherapy was 1.0 year (0.1-2.1 years).

3.6 Discussion

Herein, the clinical characteristics of the WT1 negative study cohort comprising all low grade EnOC and high grade carcinomas stratified by p53 IHC expression are described. All high grade carcinomas had either endometrioid, high grade serous or undifferentiated histology as they display significant morphological overlap with poor diagnostic inter-observer variation when diagnosed based on histology alone [229]. Due to the presence of this heterogeneous group of high grade carcinomas and its likely impact on survival, data from both the whole WT1 negative cohort as well as from classical low grade EnOC were reported separately.

Overall, the rates of endometrioisis [209, 271] and synchronous endometrial carcinomas were consistent with the literature [129, 231]. The presence of endometrioisis and endometrial carcinomas were largely based on pathology reports and as such this data was not collected in a systematic way, posing a limitation in evaluating their prognostic value. In line with the published data [226, 274, 281], the majority of patients in our cohort presented with early stage disease with favourable five year DSS of over 90% in stage I disease for both cohorts. Interestingly, DSS for stage II disease in the classical EnOC cohort was similarly favourable, approaching nearly 90% at five years. Notably, a fifth of early stage patients did not undergo full surgical staging with the majority due to the omission of an omentectomy. Furthermore, most patients did not undergo systemic lymph node sampling and none underwent lymphadenectomy. This may suggest that particularly in stage I disease, less radical surgery may be appropriate and that second surgeries for comprehensive staging could potentially be avoided. This finding is in keeping with Kobel et al in which the ten year DSS for 'apparent' stage IA and IB EnOC was 96% despite no patients undergoing an omentectomy or lymph node sampling [281]. This is also in line with several large studies demonstrating the extremely low rate of lymph node metastases in 2% or less in early stage EnOC [284-286].

In stage 1 disease, ten year RFS and DSS were lower than reported in the literature for stage IA/IB disease at just over 80% however this is likely due to the presence of high grade carcinomas in nearly 20% of this cohort. In the stage IC cohort which were comprised of mostly low grade EnOC, ten year DSS was favourable at just over 90%. When those who received adjuvant platinum chemotherapy were compared to those who did not in stage IC disease, there were no significant differences in RFS or DSS. Whilst the numbers were small in each cohort thus limiting the statistical power of this comparison, these results are consistent with the large study of 3552 stage I EnOC performed by Oseledchyk et al which found no survival benefit with adjuvant chemotherapy in stage IA/IB disease of any grade, and stage IC low grade (grade 1 and 2) disease [291].

In this study, ten year RFS and DSS rates were lower than that observed at five years in early stage disease, but not in advanced stage. This was particularly evident in stage II disease with approximately 10% of ovarian cancer recurrences and deaths occurring beyond five years. This same pattern was also observed in classical low grade EnOC tumours. This is akin to the study by Parra-Herran et al of EnOC where the separation of DSS curves between FIGO grade 1 and 2 EnOC only occurred at five years illustrating late relapses in grade 2 EnOC [221]. Similarly, this is mirrored by a retrospective study

of 2233 patients with endometrial carcinoma performed by Takahashi et al [401]. In this study, late recurrences (beyond five years from initial diagnosis) were associated with endometrioid carcinomas of low or intermediate grade with no lymphovascular invasion [401]. EnOC frequently express ER, and this clinical behaviour observed in early stage disease is akin to that of ER positive breast cancers which, unlike its ER negative counterpart, also demonstrate late recurrences [402]. To my knowledge, these findings have never been reported in EnOC, and have implications for the duration of clinical follow up as these patients are usually discharged after five years. Clinical trials performed in EnOC may need to consider extended follow up as late events are more likely to occur in this subtype which contrasts with HGSOC where the vast majority of relapses occur in the first 2 years [31].

Patterns of relapse in EnOC were also evaluated in this study. Nearly half of recurrences from early stage EnOC presented as a solitary pelvic mass, and all of these patients had low grade EnOC at diagnosis. This is very similar to CCOC, a histological subtype which shares common histogenic precursors with EnOC. In Macrie et al, 25% of 56 CCOC experienced pelvic relapse at first recurrence [185]. In those with early stage disease, this frequency rose to 62%. There has also been published retrospective studies exploring the role of adjuvant radiotherapy in early stage CCOC. In a study by Hoskins et al of 241 patients with CCOC, half the cohort received 3 cycles of adjuvant carboplatin and paclitaxel, followed by abdominopelvic irradiation (22.5Gy in 10 fractions over 2 weeks to the pelvis, followed by 22.5Gy to the whole abdomen and pelvic in 22 fractions over 4.5 weeks), whilst the other half received 6 cycles of conventional carboplatin and paclitaxel [184]. No disease free survival benefit for radiotherapy was observed in stage IA and IC (rupture only) disease. In the remaining stage IC/II cohort (positive cytology, surface involvement or unknown status), radiotherapy resulted in a 20% improvement in five year disease free survival (RR 0.52 (95% CI 0.33-0.95; P=0.02). It also showed that pelvic relapses were more common in patients who did not undergo radiotherapy compared to those who did (76% versus 62%). Prospective clinical trials of adjuvant radiotherapy in CCOC are now being proposed to validate these findings. Notably, this is also similar to endometrial carcinomas in which EnOC bears close histological and molecular semblance to. The vast majority of relapsed endometrial carcinomas are vaginal or pelvic recurrences. Adjuvant external beam radiotherapy has been shown to reduce the risk of local relapse in high intermediate risk endometrial carcinoma (two out of three risk factors: grade 3; age 60 years or older and deep myometrial invasion) [403].

Only one retrospective study has explored the role of adjuvant radiotherapy in EnOC [274]. As a follow up study performed in the same Canadian centre as Hoskins et al, Kumar et al performed the same comparison (chemotherapy versus chemotherapy/radiation) in 172 early stage EnOC. Here, the authors conclude that a non-statistically significant trend in disease free survival benefit from radiotherapy was observed in patients older than 55 with stage IC based on positive cytology, surface involvement or any stage II disease (n=32) (RR 1.77(95% CI 0.74-4.24). These findings reported by Kumar et al may be explained by the high propensity for isolated pelvic recurrences in early stage low grade EnOC observed in this study. This raises the hypothesis that a subset of early stage low grade EnOC may also benefit from adjuvant radiotherapy akin to CCOC.

In this study, the platinum sensitivity of platinum naïve tumours or of those which relapsed 12 months or more from the dose of last platinum was reported. The majority of this cohort received single agent platinum and the radiological response rate was nearly 50% and CA125 response rate of just over 70%. This is numerically lower than that reported in HGSOC although a matched comparison would be required to formally determine this [42]. This data is somewhat comparable to the three studies performed to date evaluating platinum sensitivity specifically in EnOC. Dawn et al found radiological responses rates of 60% in EnOC and CA125 response rates of 66% [272]. However, this study contained 56% of grade 3 carcinomas and did not undergo contemporary pathology review, as such it is likely that a good proportion of these poorly differentiated tumours were misdiagnosed HGSOC which limit the interpretation of this study. Similarly, Kline et al reported a 72% platinum response rate in 145 historically diagnosed EnOC in 1990 [298], although the proportion of grade 3 EnOC was not known. In Soovares et al, 249 patients with pathology reviewed EnOC had platinum response evaluated after primary surgery and adjuvant chemotherapy based on gynaecological examination, vaginal ultrasonography, CA125 measurement, and/or computed tomography imaging [275]. Overall complete response was 79% although this methodology is highly flawed given the heterogeneous assessment of response. Furthermore, they did not restrict the response analysis to those with measurable residual disease post-operatively thus casting some doubt on these high response rates observed.

The radiological response rate of platinum based chemotherapy in classical low grade EnOC was 50% with a similar CA125 response rate of 60%. Although the numbers in our cohort were small, this does suggest that classical low grade EnOC demonstrates moderate platinum sensitivity which is in contrast to LGSOC with low response rates of 5-10% [160].

When the survival analysis was restricted to patients with advanced stage classical low grade EnOC (the majority of which presented with stage III disease), the median DSS was over seven years with a five year and ten year DSS of 50%. This is superior to reported outcomes of advanced stage HGSOC with ten year DSS of 15% [404]. In keeping with this, Rambau et al reported a five year DSS of 59.6% for pathology reviewed stage III EnOC, the majority of which were low grade EnOC. Similarly, this is akin to the large GCIG study of seven randomised clinical trials which found stage III/IV EnOC (n=646) to have a median OS of 50.9 months [203]. Similarly, it also mirrors the indolent clinical behaviour of LGSOC with a reported medial survival of 82 months [84]

However, when accounting for the whole WT1 negative cohort, patients with stage III and IV disease were found to have a poor prognosis, particularly in stage IV disease with median RFS and DSS of less than 1 year with no survivors at five years. This may be accounted for by a quarter of patients not receiving any chemotherapy. Although the number of patients were small, it is worth noting that the majority of patients with stage IV disease who received platinum chemotherapy were platinum refractory, a finding which contrasts with first line platinum response rates of 80% in HGSOC [31].

It is also observed in this small dataset that more than 70% of stage IV patients had visceral metastases at diagnosis as well as two patients with unusual sites of disease (one had bone metastases and the other had splenic, renal and thyroid metastases diagnosed at post mortem shortly after diagnosis).

These findings are notable as both bone and thyroid metastases in EOC are extremely rare [405]. This disease distribution, together with the predominantly platinum refractory nature of this cohort, may suggest that stage IV EnOC, in particular those of high grade and p53 mutant IHC expression, is biologically distinct from HGSOC, and warrants alternative therapeutic strategies.

Eight patients with late isolated pelvic or nodal relapse in this study underwent an attempt at secondary surgery with most undergoing successful complete macroscopic cytoreduction. Interestingly, more than half these patients did not experience further recurrences after a median follow up time of over ten years which suggests that secondary surgery may have been a curative procedure. These results are in line with the DESKTOP III trial which demonstrated a PFS benefit of surgery over chemotherapy for platinum sensitive relapsed disease although OS data remains immature [66]. This data supports the use of surgical resections for late solitary relapses should be favoured over the use of chemotherapy in EnOC.

There are a few limitations of this clinical characterisation study. Firstly, over 200 samples were unable to be retrieved for pathology review which may have contributed to selection bias. The inclusion of WT1 negative tumours with high grade serous and undifferentiated carcinoma morphology are likely to have contributed to poorer outcomes. In view of this, the classical low grade EnOC cohort was characterised separately and the overall clinical profile was in keeping with that reported in the literature on EnOC.

The assessment of platinum sensitivity in this cohort was challenging with only a small proportion evaluable for radiological and CA125 response. This was due to low relapse rates and infrequent use of radiological monitoring in this cohort. RECIST criteria was unable to be employed due to variable reporting methods used and relied on the interpretation of the reporting radiologist to determine response. However, radiological response was only recorded if a 30% or more reduction in tumour diameter was reported, otherwise this was recorded as stable disease in the absence of reported disease progression. It is therefore unlikely that the rates of radiological responses to platinum chemotherapy in this study are overestimated. It is also worth noting that half these responses were evaluated in relapsed disease. At least two patients who initially presented with low grade EnOC recurred as high grade carcinomas. Whilst recurrent tumours did not undergo pathology review, it is plausible that a proportion of tumours underwent malignant high grade transformation, an extremely rare phenomenon that has been observed in LGSOC [406, 407]. As such, it is hypothesised that responses to platinum chemotherapy in this cohort may in part be a reflection of this.

3.7 Conclusion

In this study, the clinical characteristics of classical low grade EnOC as well as the whole WT1 negative cohort including high grade carcinomas following comprehensive pathology review are described. The key messages are as follows:

- EnOC is associated with a good prognosis in early stage disease.
- Late relapses beyond five years are frequent particularly in stage II disease and may thus influence follow up time in these patients.
- An isolated pelvic mass is the most common pattern of relapse and as such there may be an argument for designing clinical trials to investigate the role of adjuvant radiotherapy in EnOC akin to CCOC.
- The use of secondary cytoreductive surgery is associated with prolonged disease free survival when employed in EnOC with late solitary recurrences.
- Stage IV EnOC are biologically aggressive tumours which may be platinum refractory and are associated with very poor prognosis. Whole exome sequencing data of these tumours may help inform whether these tumours have actionable mutations which may be suitable for the development of novel therapeutic strategies.

In conclusion, I have presented data on EnOC which may have relevance to clinical management and follow up of this rare histological subtype.

4. Hormone receptor expression and endocrine sensitivity in EnOC

4.1 Introduction

As discussed in section 1.4.5, the majority of EnOC express ER and PR as demonstrated by the large Ovarian Cancer Tissue Consortium study performed by Sieh et al [81]. These findings were confirmed in an independent study by Rambau et al of contemporarily defined EnOC utilising IHC [255]. In both studies, any expression of ER and PR, as determined by the percentage of tumour nuclei stained, was found to be prognostic in EnOC. Notably in Rambau et al, the prognostic effect of ER and ER/PR co-expression was diminished when restricted to early stage disease, with PR losing significance. In contrast, the frequency of AR expression in EnOC has only been evaluated in small studies and is variable depending on the IHC expression threshold used [83, 87, 90, 94]. Furthermore, its prognostic role in EnOC is unknown.

In view of its high frequencies of ER expression, EnOC is considered an endocrine sensitive disease. Endocrine therapy is not licensed for use in EOC due to conflicting data derived from more than 50 phase II clinical trials. These heterogeneous trials were mostly performed in heavily pre-treated patients of all histological subtypes of EOC, comprising both ER positive and negative disease [295] (Appendix C). Furthermore, different thresholds and methods of measuring ER expression were also used. The only phase III clinical trial performed comparing tamoxifen against conventional chemotherapy in a platinum resistant ovarian cancer population, failed to demonstrate a survival advantage [110]. As a result, the use of endocrine therapy varies worldwide and is not considered standard of care.

There is however good prospective [112, 113] and retrospective data [295] show that the degree of ER expression predicts for endocrine sensitivity in EOC. These studies utilised the histoscore, a weighted scoring method which accounts for stain intensity and percentage of tumour nuclei stained, as the preferred method of determining ER expression. An ER histoscore of 150 or greater was found to be the threshold at which patients derived benefit from endocrine therapy in the prospective trials [112, 113]. In the large retrospective study performed by Stanley et al of endocrine therapy in recurrent HGSOC, patients with a treatment free interval of ≥180 days and a tumour ER histoscore of >200 derived the greatest benefit [295] (Appendix C).

As previously discussed, the five main histological subtypes of EOC are recognised to be biologically and clinically distinct. Apart from EnOC, the Ovarian Cancer Tissue Consortium study found LGSOC and HGSOC to have the highest levels of ER expression [81]. There is emerging retrospective data supporting the use of maintenance endocrine therapy in LGSOC in both the relapsed [118] and first line settings [119]. In addition, a small retrospective study performed by Heinzelmann-Schwarz et al demonstrated improved recurrence free survival in patients with HGSOC who received maintenance letrozole compared to those who did not. [120]. Together, these studies support the evaluation of endocrine therapy in EOC in a histological subtype- specific manner. As discussed in section 1.4.9.3, there is minimal published data describing the endocrine sensitivity of EnOC which is in contrast to HGSOC and LGSOC [222, 296].

In this chapter, the hormone receptor expression of ER, PR and AR are described as weighted histoscores, and correlated to RFS and DSS, in both the earlier defined WT1 negative cohort as well as in the classical low grade EnOC group. The endocrine sensitivity of EnOC is also evaluated.

4.2 Results

4.2.1 Hormone receptor expression in EnOC

Overall, 103 (82.4%), 103 (82.4%) and 107 (85.6%) of cases were evaluable for ER, PR and AR, respectively. 77.7%, 74.8% and 70.1% expressed any ER, PR and AR, respectively. The spread of ER, PR and AR expression was similar for both the WT1 negative and classical low grade EnOC cohorts (Figure 26). All hormone receptors were non-normally distributed and PR expression demonstrated a bimodal distribution (Hartigan's dip test; P<0.0001). There was some evidence of multimodality for AR expression (Hartigan's dip test; P=0.0374) in the classical low grade EnOC cohort. The majority of tumours had low AR expression (histoscore 0-50) (76.6% WT1 negative cohort, 77.0% classical low grade EnOC).

In ER and PR, histoscores were grouped according to strong (histoscore>150) or weak (histoscore≤150) expression. There were significant associations between ER and PR (rho=0.68, P<0.0001), ER and AR (rho=0.46, p<0.0001), with weaker but significant associations between PR and AR (rho=0.38; P<0.0001). The frequency of strong ER was higher in the strong PR group compared to the weak PR group (59.6% vs 20.8%, p<0.0002).

Figure 27 illustrates the distribution of strong and weak ER and PR. In both the WT1 negative and classical low grade EnOC cohorts, the highest frequencies were that of strong ER/strong PR and weak ER/weak PR (WT1 negative cohort: 28.0% and 42.0%, respectively, classical low grade EnOC: 36.8% and 27.9%, respectively).

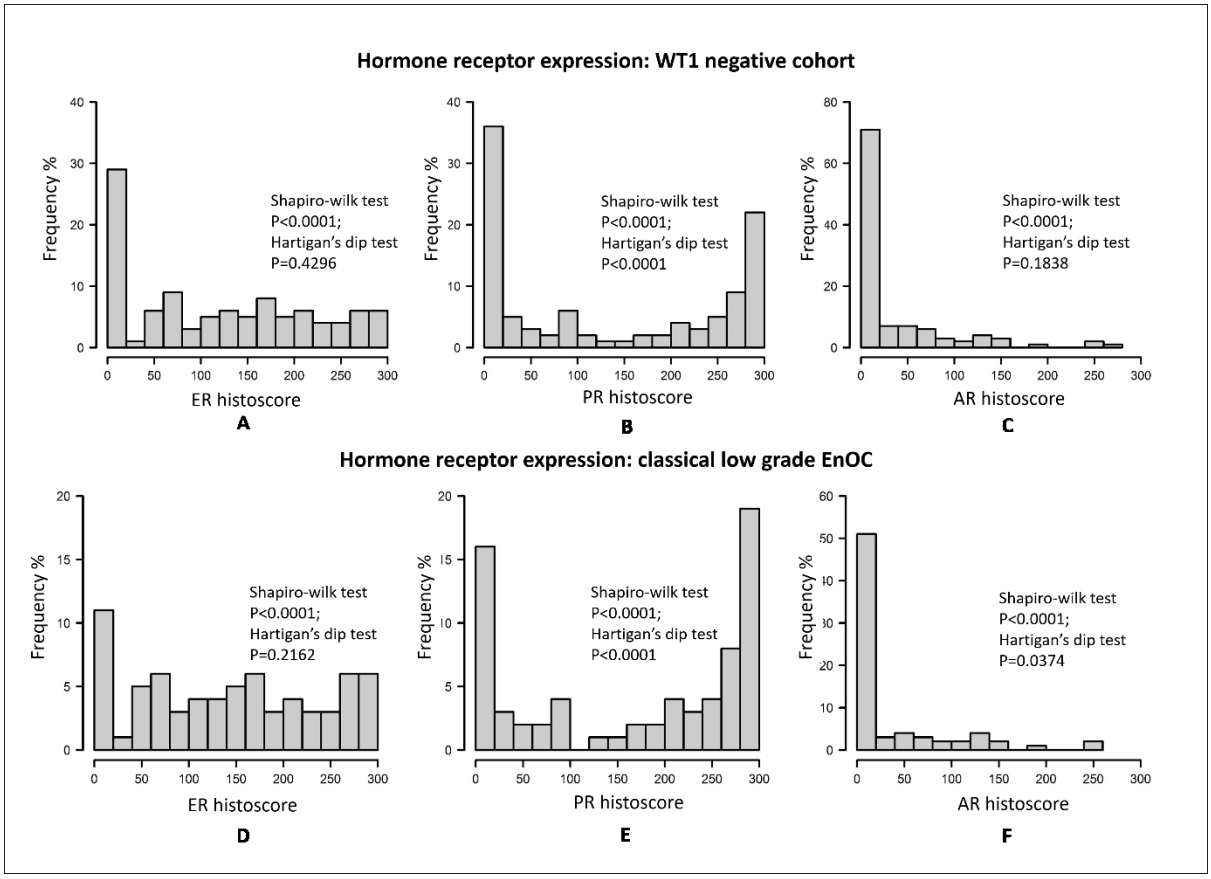

Figure 26: Spread of ER, PR and AR immunohistochemical expression in the WT1 negative cohort (A, B and C, respectively) and classical low grade EnOC cohort (D, E and F, respectively). Figure by Dr Robb Hollis.

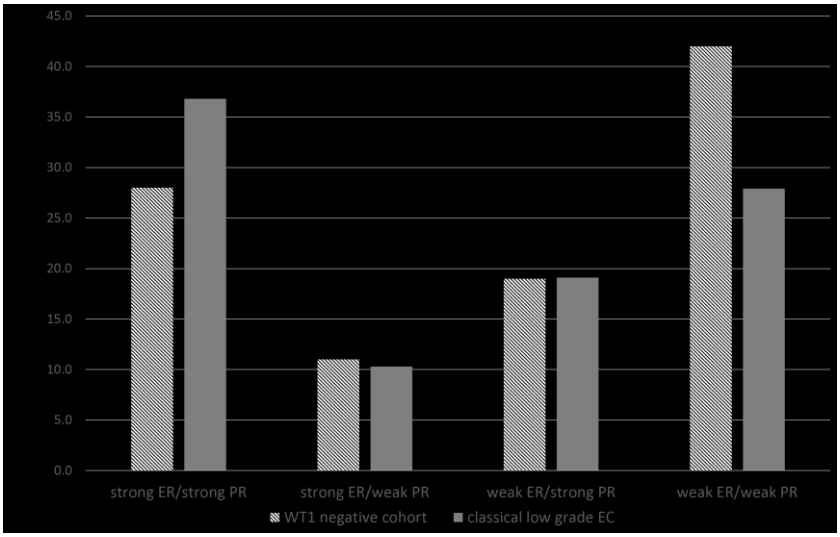

Figure 27: Distribution of strong (histoscore>150) and weak (histoscore≤150) ER and PR in the WT1 negative cohort and classical low grade EnOC cohort.

4.2.2 Hormone receptor expression and survival

WT1 negative cohort

Strong PR was significant for both RFS (univariable HR 0.24 (95%CI 0.10-0.55); P<0.001) and DSS (univariable HR 0.14 (95%CI 0.05-0.39); P<0.001) when compared to weak PR. Strong ER was significant for DSS (univariable HR 0.37 (95%CI 0.15-0.91); P=0.03) and approached significance for RFS (univariable P=0.07) when compared to weak ER.

Strong PR expression remained an independent predictor of DSS (multivariable HR 0.23 (95%CI 0.08-0.72); P=0.011) and approached significance for RFS (multivariable P=0.092), but strong ER lost significance for both RFS (P=0.712) and DSS (P=0.299) upon multivariable analysis (Table 16, Figure 28). Ten year RFS and DSS for strong PR was 82.6% and 91.4% compared to 47.8% and 50.7% for weak PR, respectively. The differential impact of PR on DSS was most marked in stage II disease (HR0.10 (95%CI 0.13-0.82); P=0.031) (Figure 29). AR expression was not associated with RFS or DSS on univariable or multivariable analysis.

When compared with weak PR, tumours with strong PR were more likely to be diagnosed with low grade disease (P<0.001), early stage disease (P=0.018), and were more likely to be surgically cyto-reduced to <2cm (P=0.042) (Table 17). No differences in age, BMI, year of diagnosis, and proportion of patients receiving at least three cycles of platinum chemotherapy were observed between the two cohorts.

Classical low grade EnOC cohort

In the classical low grade EnOC cohort, only strong PR was independently significant for DSS (multivariable HR 0.13 (95%CI 0.02-0.64); P=0.013) and approached significance for RFS (multivariable HR 0.34 (95%CI 0.09-1.22); P=0.097) (Table 16). Ten year RFS and DSS for strong PR was 82.9% and 93.1% compared to 58.7% and 60.2% for weak PR, respectively. No significant differences in RFS or DSS were observed for ER or AR on univariable or multivariable analysis (Table 16).

Table 16: Univariable and multivariable analysis of hormone receptor expression and survival in the WT1 negative cohort and classical low grade EnOC cohort.											
WT1 negative cohort (n=125)											
		Univariable						**Multivariable[a]**			
				RFS		**DSS**		**RFS**		**DSS**	
		N	**%**	**HR (95% CI)**	**P**	**HR (95% CI)**	**P**	**HR (95% CI)**	**P**	**HR (95% CI)**	**P**
ER	>150	41	39.8	0.50 (0.23-1.07)	0.072	0.37 (0.15-0.91)	0.03	0.84 (0.34-2.08)	0.712	0.57 (0.2-1.64)	0.299
	≤150	62	60.2	ref		ref		ref		ref	
PR	>150	47	45.6	0.24 (0.10-0.55)	<0.001	0.14 (0.05-0.39)	<0.001	0.45 (0.18-1.14)	0.092	0.23 (0.08-0.72)	0.011
	≤150	56	54.4	ref		ref		ref		ref	
AR	0-100	94	87.9	ref		ref		ref		ref	
	101-200	10	9.3	0.83 (0.26-2.72)	0.764	0.61 (0.15-2.58)	0.506	1.01 (0.28-3.65)	0.988	0.46 (0.09-2.28)	0.341
	201-300	3	2.8	1.41 (0.19-10.35)	0.737	2.07 (0.28-15.39)	0.479	2.88 (0.28-29.96)	0.376	3.35 (0.28-40.39)	0.341
classical low grade EnOC (n=87)											
				HR	**P**	**HR**	**P**	**HR**	**P**	**HR**	**P**
ER	>150	33	47.1	0.63 (0.21-1.88)	0.407	0.45 (0.12-1.69)	0.236	0.41 (0.1-1.68)	0.215	0.26 (0.05-1.44)	0.122
	≤150	37	52.9	ref		ref		ref		ref	
PR	>150	42	60	0.39 (0.14-1.10)	0.076	0.18 (0.05-0.66)	0.01	0.34 (0.09-1.22)	0.097	0.13 (0.02-0.64)	0.013
	≤150	28	40	ref		ref		ref		ref	
AR	0-100	63	85.1	ref		ref		ref		ref	
	101-200	9	12.2	1.76 (0.50-6.19)	0.378	1.38 (0.3-6.23)	0.678	NE	NE	NE	NE
	201-300	2	2.7	NE	NE	NE	NE	NE	NE	NE	NE

[a]adjusted for stage, residual disease, decade of diagnosis and age.
Legend: RFS=relapse free survival; DSS=disease specific survival; ER=oestrogen receptor; PR=progesterone receptor; AR=androgen receptor; EnOC=endometrioid ovarian carcinoma; ref=reference, NE=not evaluable.

Table 17: Clinical characteristics of strong (histoscore>150) and weak (histoscore≤150) PR expression.					
	PR>150 (n=47)		PR≤150 (n=56)		P-value
	N	%	N	%	
median age	56(28-88)	NA	60(32-79)	NA	0.105
BMI[a]	24.9	NA	24.8	NA	0.545
Endometrial cancer	6	12.8	9	16.1	0.847
Endometrioisis	17	36.2	15	26.8	0.417
Grade					
1	39	83.0	26	46.4	<0.001
2	4	8.5	20	35.7	
3	4	8.5	10	17.9	
Year of Diagnosis		0.0			
1980s	7	14.9	8	14.3	0.509
1990s	16	34.0	27	48.2	
2000s	14	29.8	12	21.4	
2010s	10	21.3	9	16.1	
Stage					
I	21	46.7	21	37.5	0.018
II	21	46.7	20	35.7	
III	3	6.7	9	16.1	
IV	0	0.0	6	10.7	
UK	2	NA	0	NA	
Surgical Cytoreduction					
<2cm[b]	43	95.6	43	79.6	0.042
≥2cm	2	4.4	11	20.4	
UK	2	NA	2	NA	
At least 3 cycles platinum					
Yes	30	63.8	39	69.6	0.679
No	17	36.2	17	30.4	

[a]Evaluable in 25 cases for PR>150 and 34 cases for PR≤150. [b]32 cases were surgically cyto-reduced to 0cm. Classification of optimal surgical cytoreduction changed over time; some of the patients were diagnosed at a time when resection to <2cm was considered optimal.
Legend: BMI=body mass index.

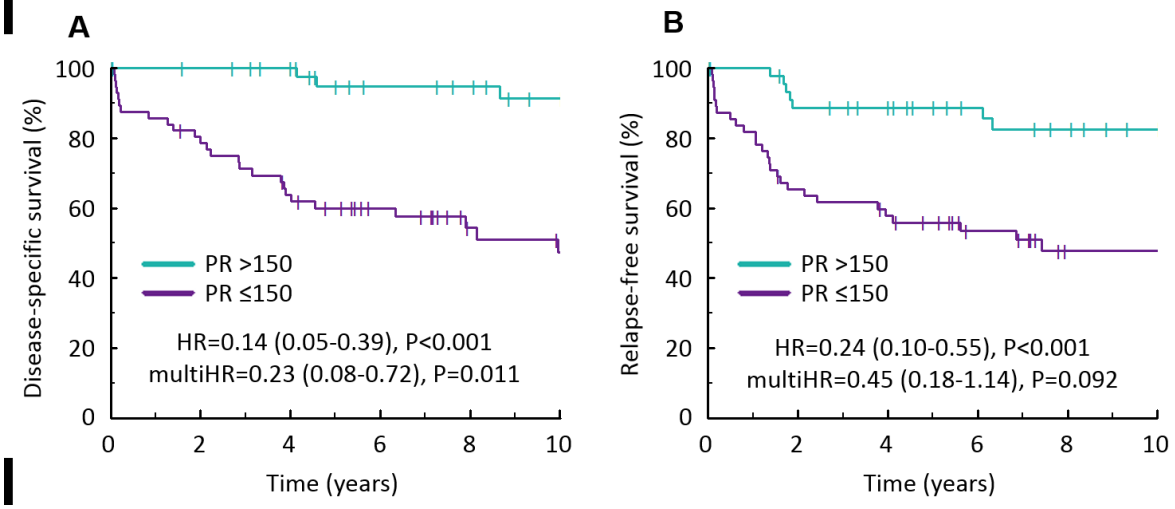

Figure 28: Disease specific survival (A) and relapse free survival (B) by strong (histoscore >150) and weak (histoscore ≤150) PR expression.

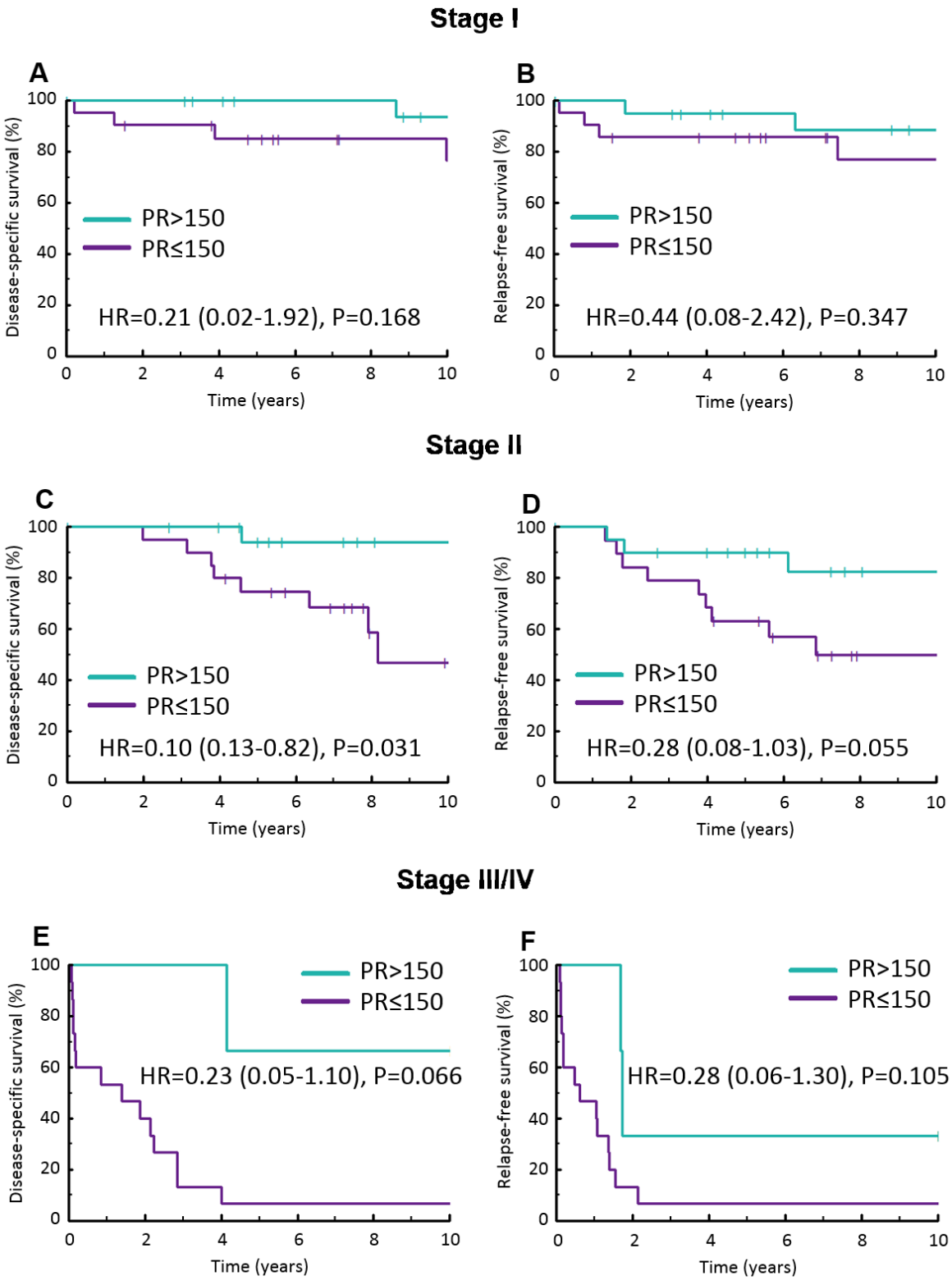

Figure 29: Kaplan Meier survival curves for disease specific survival (A, C, E) and relapse free survival (B, D, E) for PR expression by stage.

4.2.3 Endocrine sensitivity in EnOC

Of the 125 WT1 negative tumours, 11 (8.8%) patients received endocrine therapy (Table 18). Of these, eight (72.7%) patients had classical low grade EnOC at diagnosis. A total of 13 courses of endocrine therapy courses were delivered (letrozole (n=7), megesterol acetate (n=3), medroxyprogesterone (n=2), tamoxifen (n=1)). 11 were used as treatment following disease progression (one CA125 only relapse), one as maintenance treatment due to chemotherapy toxicity, and one as adjuvant treatment following surgical cytoreduction to <2cm for stage III disease. Nine (81.8%) of 11 evaluable courses had an ER histoscore of 150 or greater, whilst seven (77.8%) of nine evaluable courses had PR histoscores less than 100.

Of the eight evaluable courses of endocrine therapy with a duration of least four weeks started as treatment for relapsed disease, the median duration of therapy was 317 days (range 35-615 days). Of these eight courses of endocrine therapy, the median interval from last platinum chemotherapy received to endocrine therapy initiation was 322 days (range 9-2233 days). Of the five evaluable CA125 responses, three had PR, one had SD, and one displayed delayed stabilisation of CA125. Two patients had evaluable radiological responses (1 PR, 1 SD). Notably, one patient whose tumour was negative for both ER and PR expression, received megesterol acetate for CA125 only relapse for 594 days, followed by sequential tamoxifen due to CA125 progression for another 385 days. Best CA125 response with each endocrine therapy was PR.

Table 18: Treatment summary for endocrine therapy received.

Pat.	ET	Path	p53 IHC	ER[b]	PR[b]	DOT/ days	TFI/ days	Start reason	Stop	Prior lines of chemo	Ca125	Radio.
484	Prov	G1 EnOC	wt	NE	NE	UK	166	PD on non-platinum	UK	2	SD	NE
1893	Prov	G1 EnOC	wt	NE	NE	729	NA	Adjuvant post surgery for stage III	EOT	0	NE	NE
7713	Let	G1 EnOC	wt	250	249	615	539	Relapse post platinum	PD	1	PR	NE
8155	Meg	G2 EnOC	wt	288	300	1076	25	Maint. following platinum toxicity	UK	2	NE	NE
8624	Meg	G1 EnOC	wt	150	0	4	13	Relapse post platinum	Death	2	SD	PD
9420	Let	G1 EnOC	wt	198	40	477	322	Relapse post platinum	Toxicity	2	NE	PR
9710[a]	Meg	G1 EnOC	wt	0	0	594	818	CA125 only relapse post platinum	CA125 PD	1	PR	NE
9710[a]	Tam	G1 EnOC	wt	0	0	385	NA	CA125 PD on Meg	CA125 PD	1	PR	NE
21729	Let	G2 EnOC	mut	256	NE	222	110	Relapse post platinum	PD	1	Delay SD	NE
21763	Let	G1 EnOC	wt	165	93	249	2233	Relapse post platinum	PD	1	NE	NE
21770	Let	G2 EnOC	mut	270	NE	35	9	Progression on platinum	Death	1	NE	NE
21899[a]	Let	HGS	mut	225	0	120	77	PD on platinum	PD	2	SD	PD
21899[a]	Let	HGS	mut	225	0	UK	90	PD on weekly taxol	UK	3	NE	NE

[a] duplicate patient.
[b] histoscore
Legend: Pat=patient; ET=endocrine therapy; prov=provera (medroxyprogesterone acetate); let=letrozole; meg=megesterol acetate; tam=tamoxifen; G=grade; EnOC=endometrioid ovarian carcinoma; HGS=high grade serous morphology; wt=wild-type expression; mut=mutant expression; chemo=chemotherapy; path=pathology; IHC=immunohistochemistry; ER=oestrogen receptor; PR=progesterone receptor; DOT=duration of therapy; TFI=treatment free interval (from last chemotherapy); Radio=radiological response; PD=progressive disease; SD=stable disease; PR=partial response; NE=not evaluable.

4.3 Discussion

In this study, the majority of EnOC display ER and PR expression which is in keeping with the literature [81, 255]. In particular, PR expression displays a bimodal distribution unlike the expression of ER. In order to ascertain if this effect was in part due to the inclusion of high grade carcinomas and low grade EnOC with p53 mutated expression on IHC, both factors which may influence hormone receptor expression patterns, the analyses was restricted to the pathologically homogenous cohort of classical low grade EnOC which demonstrate the same findings. This contrasts with ER positive breast carcinomas which demonstrate a bimodal distribution of ER but uniform distribution of PR [408]. The bimodal distribution of PR in EnOC may also suggest bimodal gene expression akin to that of ER in breast cancer [409, 410]. To my knowledge, the distribution of ER and PR expression as weighted histoscores has never been formally described in EnOC or EOC before. When compared to the study performed by Rambau et al of 182 pathology reviewed EnOC, frequencies for ER staining were 12.7%, 14.3%, and 73% and that of PR were 13.3%, 12.7%, and 74% for negative (0% nuclear stain), weak (1-50% nuclear stain) and strong expression (>50% nuclear stain), respectively [255]. When compared to the classical low grade EnOC cohort, the rates of ER and PR negativity were similar, whereas the rates of strong ER and strong PR in our study were numerically lower at 47.1% and 60.0%, respectively. These observed differences may be in part due to our use of a weighted histoscore, which in addition to percentage nuclei stained, also incorporates nuclear stain intensity.

In the WT1 negative cohort, strong PR expression, when compared to weak PR, is associated with DSS independent of stage, residual disease, decade of diagnosis and age. A trend for significance was observed for RFS. In contrast, strong ER was significant on univariable analysis but was not an independent variable of prognosis. When this analysis was restricted to the classical low grade EnOC, strong PR remained an independent predictor of prognosis whereas no correlation was observed between strong or weak ER in the univariable or multivariable analysis for RFS or DFS. These findings parallel that of HGSOC in which strong PR (>50% nuclei staining), but not weak PR (1-50% nuclei staining), expression was found to be an independent predictor of prognosis in HGSOC [81].

Sieh et al and Rambau et al were the two largest studies performed evaluating the prognostic role of hormone receptor expression in EnOC [81, 255]. In Rambau et al, EnOC underwent contemporary pathology review utilising IHC. Both studies found that any ER or PR expression were independently associated with DSS. No survival differences were observed between the weak (1-50%) and strong (>50% nuclear stain) expression cohorts. These differences to our study may be explained by our scoring methodology, as well as the use of weak expression rather than negative expression as a comparison cohort. The histoscore threshold of 150 was chosen due to the spread of PR and ER expression in our study. Furthermore, the prospective studies performed by Bowman and Smyth et al established an ER histoscore of 150 as a treatment threshold for the use of endocrine therapy [112, 113]. In Stanley et al, patients with HGSOC and ER histoscores of 250-300 received endocrine therapy for a longer duration then compared to those with ER histoscores of 0-150, further demonstrating that the degree of ER expression predicts for endocrine sensitivity [295]. As such, it is hypothesised that the histoscore may provide greater granularity at higher expression levels in determining prognosis. A

formal comparison of scoring methods utilising receiver operating characteristics analyses would be required to confirm this.

In the overall WT1 negative cohort, the independent impact on DSS of strong PR was most marked in stage II disease with DSS of over 90% in this cohort. Although most patients with stage II disease received platinum chemotherapy, no significant differences were observed in the proportion of patients who received at least three cycles of platinum chemotherapy between the strong PR and weak PR cohorts. It thus raises the hypothesis as to whether patients diagnosed with early stage EnOC and strong PR expression, could afford to have adjuvant chemotherapy omitted even in stage II disease. This finding contrasts with the study performed by Rambau et al where PR lost significance and ER was of only borderline prognostic significance when restricted to early stage disease [255]. It is possible that the use of the histoscore in determining the degree of hormone receptor expression and the greater power of this study explain these differences. Together with the bimodal distribution of PR, this data supports the use of a PR histoscore>150 as a reproducible biomarker which can be used to identify a cohort of patients with superior prognoses. This finding, together with the significant association of strong ER and PR, is also in keeping with the role of PR as surrogate for a functionally intact ER pathway and its role in inhibiting cell proliferation [80].

AR expression was also evaluated in both cohorts. Overall, AR expression was low with the majority of cases negative or with very weak expression (histoscore1-50) for AR. The degree of AR expression was not associated with survival which contrasts with HGSOC however, the numbers with higher expression of AR were very small which limit the interpretation of these findings. Nonetheless, no other studies have evaluated the prognostic significance of AR specifically in EnOC and the overall weak AR expression displayed in EnOC thus makes it an unreliable biomarker. Jonsson et al found a trend for OS with co-expression of PR and AR in a sub-group analysis of 31 EnOC, however notably post-operative residual disease was not recorded in this study [83]. In contrast, AR expression has been shown to have independent prognostic significance in retrospective studies of HGSOC [96, 104].

In the largest series reported to date, the endocrine sensitivity of EnOC, most of which were low grade, was evaluated. Patients who received endocrine therapy for relapsed disease had a median duration of therapy of nearly a year. This contrasts with HGSOC where median duration of endocrine therapy has been shown to be approximately four months [295] (Appendix C). All five courses of endocrine therapy evaluable for CA125 response demonstrated response, stabilisation or delayed stabilisation of CA125. Similarly, radiological response and stabilisation was observed in both evaluable patients. The majority of cases had an ER histoscore of 150 or greater with most cases displaying low PR expression in keeping with ER expression as the main predictor of endocrine response as outlined in the literature [112, 113, 295]. The size of this cohort illustrates the rarity of relapsed cases of EnOC and the challenges in performing clinical trials in assessing the efficacy of endocrine therapy in EnOC. As such, this data contributes to the body of literature demonstrating activity of endocrine therapy in relapsed EnOC.

In this study, both histoscores as determined by BS and YI were averaged in order to minimise inter-observer variability. A third scorer, SH, was introduced if the difference between the histoscores were greater than 50 points, thus reducing any observer bias that may have occurred. TMAs, rather than whole tissue sections, were used for scoring as good concordance between the two have been demonstrated in the literature [411]. Less than 10% of patients received endocrine therapy and is therefore unlikely to have influenced the reported survival outcomes. Furthermore, no differences in platinum chemotherapy received were observed between the strong and weak PR cohorts, and multivariable analysis also corrected for decade of diagnosis to account for variations in management over time.

Further to this work, hierarchical clustering of PR and ER histoscores across 107 WT1 negative EnOC of all grades, excluding those with undifferentiated and high grade serous histology (Appendix D) [412]. Here, four subgroups of EnOC defined by hormone receptor expression patterns were identified (PR^{high}/ER^{high}, PR^{high}/ER^{low}, PR^{low}/ER^{high}, PR^{low}/ER^{low}). Similar to this study, both PR^{high} cohorts were independently associated with superior prognosis. Based on the unsupervised clustering algorithm, the histoscore threshold identified was around 150 to define the PR^{high} and PR^{low} groups, thus supporting the threshold used in this study.

4.4 Conclusion

This is the largest study performed to date evaluating the prognostic role of ER, PR and AR expression as weighted histoscores in contemporary defined EnOC. The use of PR histoscore>150 can define a group of patients with good prognosis, thus generating the hypothesis that these patients with early stage EnOC could potentially be spared adjuvant chemotherapy. To my knowledge, this is the largest series to date illustrating the endocrine sensitivity of EnOC, and that these patients can derive prolonged benefit from endocrine therapy. Given the prevalence of ER expression and the indolent nature of EnOC particularly in low grade disease as illustrated in section 3, there may be an argument for considering maintenance or adjuvant endocrine therapy particularly in advanced stage disease in order to improve patient outcomes. This will be comparable to the management of ER positive breast cancer, and supported by the emerging retrospective data of maintenance endocrine therapy in LGSOC and HGSOC [119, 120].

5. Single nucleotide variant and copy number analysis

5.1 Introduction

As described in chapter 1, the majority of EnOC arise from endometriosis, and most grade 1 and 2 (low grade) EnOC display a classical IHC profile comprising WT1 negativity, wild-type p53 expression, and ER positivity [226]. These tumours bear close histological and molecular resemblance to EnEC [413]. Commonly mutated genes in EnOC include *PTEN, ARID1A, PIK3CA* and *CTNNB1* [227, 247, 254]. Mutations in *KRAS*, encoding a member of the MAP kinase pathway, and MSI resulting from mutations in the MMR genes, are also associated with EnOC [227]. *TP53* mutations (*TP53*^{mut}) are rare in low grade EnOC and have been reported to occur in less than 10% of cases [228], with higher frequencies of up to 32% in all grades of EnOC [243, 254, 302].

In chapter 1, the diagnostic challenges in differentiating high grade EnOC from HGSOC on the basis of morphology alone [226, 413] are discussed. In particular, a proportion of HGSOC demonstrate solid, pseudo-endometrioid and/or transitional-cell-like growth patterns (SET pattern), which may be associated with *BRCA1* mutations [241]. De-differentiated carcinomas are rare, aggressive tumours consisting of undifferentiated carcinomas with a low grade EnOC component. They often lack defining morphologic features and can also be misdiagnosed as high grade EnOC [257, 261]. Through the refinement of EOC diagnostic criteria [242], several studies have now demonstrated that many previously diagnosed high grade ECs are in fact HGSOC [226, 243]. This is supported by gene expression profiling studies demonstrating that a proportion of high grade EnOC cluster together with HGSOC [243, 246-248]. As such, true high grade EnOC are increasingly rare, representing around 5-19% of EnOC cases [224, 226, 254]. Reports of high grade EnOC have suggested poor clinical outcomes in this subset, in contrast to their low grade counterparts [272, 282]

To date, several studies have attempted to define the molecular landscape of EnOC. The study cohorts and molecular tools used across the studies have been heterogeneous. For example, some studies were performed only in low grade EnOC [228] whereas others accounted for all grades of EnOC [227, 375]. Some studies did not describe the diagnostic criteria used [249, 254, 375, 381], and others relied on historical pathological subtyping [227, 228, 243, 246-248, 375, 414, 415]. It is thus plausible that a proportion of these cohorts comprised misdiagnosed HGSOC. More in-depth molecular analysis utilising WES and WGS comprised very small cohorts of EnOC [375, 379]. For example Teer et al performed WES in only six grade 1 and 2 stage I EnOC, and targeted sequencing in 14 EnOC of unknown grade [379]. In Wang et al, whole genome sequencing was applied to 29 EnOC once again of unknown grade [375]. As such, the molecular landscape of EnOC, in particular high grade EnOC, is not well defined.

Currently, management of EnOC follows the historic 'one size fits all' approach, with aggressive cytoreductive surgery and platinum based chemotherapy forming the mainstay of treatment. This contrasts with routine molecular stratification of HGSOC with the advent of PARP inhibitors for *BRCA1/2*-mutant cases [150, 294] and the emergence of histotype-specific management in other EOC subtypes. These include the use of MEK inhibitors in LGSOC [416] and immune check point inhibitors in CCOC [193].

WT1 IHC is a useful tool to discriminate high grade EnOC (WT1 negative) from HGSOC (WT1 positive) [229, 230, 244, 245], reducing inter-observer variation [129, 224, 229, 243].

Here, whole exome sequencing (WES) was performed to investigate the molecular landscape of WT1 negative EnOC, including high grade carcinomas of endometrioid, high grade serous and undifferentiated histology. The hypothesis of this chapter is that there are molecular subgroups with differential clinical outcomes within EnOC, and molecular biomarkers which provide greater prognostic granularity over grade. The six WT1 positive low grade EnOC, which formed a separate cohort of interest in this study, also underwent WES and it is hypothesised that these tumours are also true EnOC at the molecular level.

5.2 Results

5.3 Molecular landscape

As described in section 2.6, the WT1 negative cohort was categorised by p53 IHC expression and grade. Three cohorts were identified: group 1) WT1 negative p53 wild-type expression low grade EnOC (classical low grade EnOC), group 2) WT1 negative p53 mutated expression carcinomas (comprising both low grade EnOC and high grade carcinomas), and group 3) WT1 negative p53 wild-type expression high grade carcinomas. I chose to perform WES in all tumours of group 2 and 3, as well as a select cohort of group 1 as this group was deemed to be pathologically homogenous.

In total, 61 cases were characterised by WES. 25,067 variants were identified with a median of 214 variants per sample (range 198-3852). Six tumours were considered 'hyper-mutated' (>500 mutations per sample) and one tumour was considered 'ultra-mutated' (>3500 mutations). Five genes were mutated at high frequency (>20%) across the cohort: *TP53* (45.9%); *ARID1A* (41.0%), *CTNNB1* (31.1%), *PTEN* (24.6%) and *PIK3CA* (23.0%) [356]. The type and location of mutations were in keeping with the literature (Figure 30). Overall, 36 (59.0%) cases had one or more mutations in *PTEN*, *CTNNB1*, *ARID1A*, or *PIK3CA* (EnOC^{like} profile) [229, 356], and 25 (41.0%) tumours had no mutations in any of these genes (EnOC^{wt} profile). Other commonly mutated genes included *FBXW7* (13.1%), *KRAS* (13.1%), *APC* (11.5%), *BRCA2* (11.5%), *MTOR* (9.8%) and *PIK3R1* (9.8%).

Molecular group	**Definition**
EnOC^{like} profile	One or more mutations in *PTEN*, *CTNNB1*, *ARID1A*, or *PIK3CA*
EnOC^{wt} profile	No mutations in *PTEN*, *CTNNB1*, *ARID1A*, or *PIK3CA*
Legend: EnOC^{wt} = Endometrioid ovarian carcinoma wild type	

The majority of *TP53* mutations were missense mutations (16/28; 57.1%). The remainder were nonsense mutations (n=4), in-frame deletions (n=4), frame shift deletions (n=3), and multi-hit (frame shift insertion + missense mutation) mutations (n=1). Of 25 tumours with *ARID1A* mutations, most were nonsense (n=7) and frameshift mutations (n=10, 4 insertions, 6 deletions), two were missense

mutations and six had multiple hits. Of 15 *PTEN* mutations, nine were missense mutations, three were frameshift deletions, two were nonsense mutations, and one was a splice site deletion. Of 19 *CTNNB1* mutations, 18 were missense mutations, and one was an in-frame deletion. 17 tumours had *CTNNB1* mutations in exon 3 in line with the literature. Of 14 *PIK3CA* mutations, 13 were missense mutations.

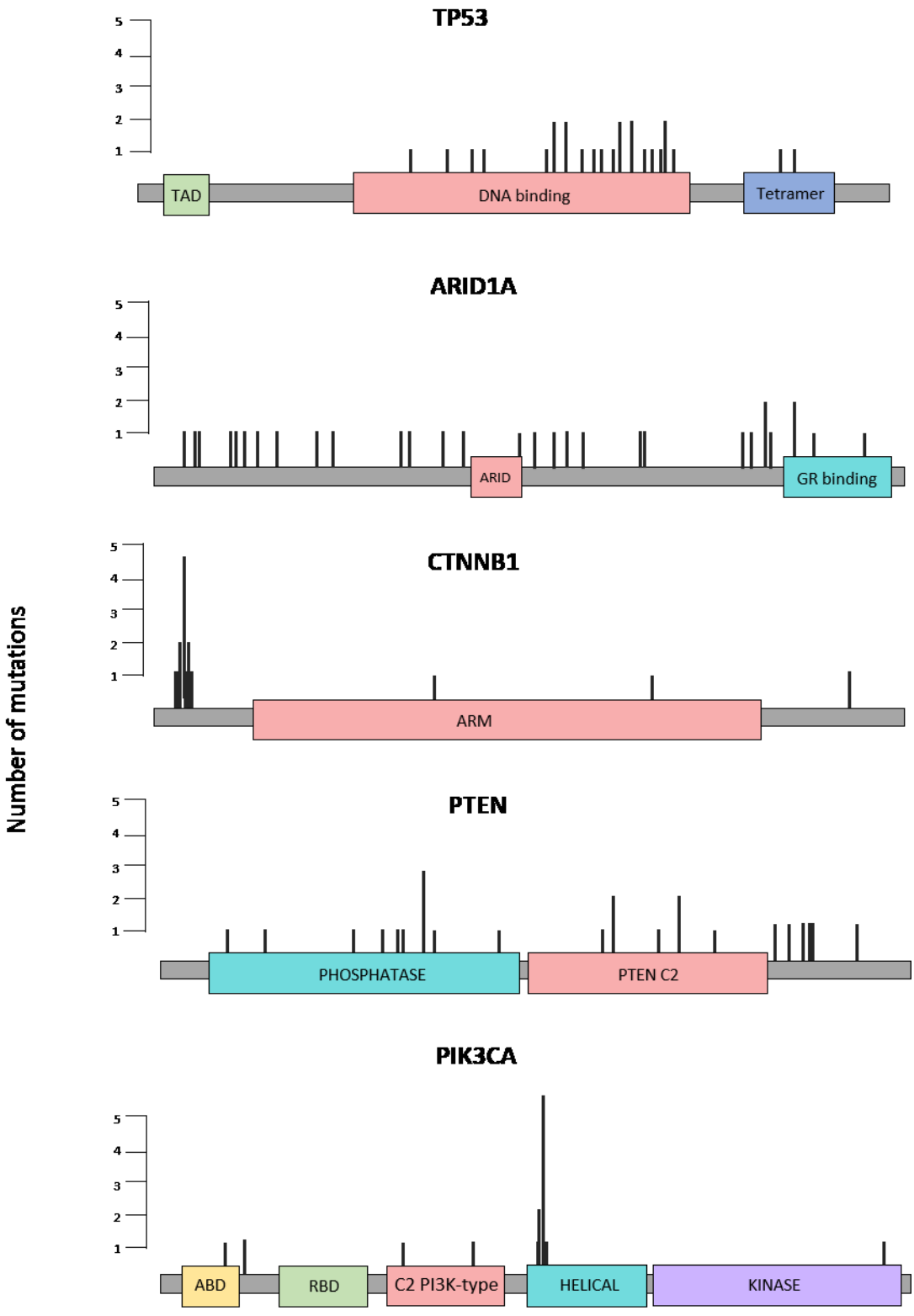

Figure 30: Lollipop plot of the 5 most commonly mutated genes in EnOC. Figure by Dr John Thompson.

5.3.1 Mutational and Immunohistochemical correlation

Correlation between mutational status and TMA IHC for MMR, beta-catenin, ARID1A and PTEN was performed (Table 19, Figure 31). For MMR, the mutation rate was 40.0% in the IHC loss group, whereas this was 8.2% in the IHC intact group (P=0.089). For beta-catenin, the mutation rate was 85.7% in the tumours which demonstrated aberrant nuclear staining, and 14.3% in normal membranous staining (P<0.001). For *ARID1A*, the mutation rate 85.7% in the IHC loss group, and 27.9% in the IHC intact group (P<0.001). For *PTEN*, the mutation rate was 71.4% in the loss group, and 5.4% in the IHC intact group (P<0.001). During the scoring process, a cohort of tumours which demonstrated heterogeneous *PTEN* staining (one core intact, one core loss) were identified. The mutation rate was higher at 40.0% in this group when compared to the IHC intact group although this difference only approached significance (P=0.063).

Table 19: Correlation of tissue microarray immunohistochemistry and mutation status.						
IHC	**Stain**	**Mut**	**WT**	**NE**	**Evaluable**	**Mutation (%)**
MMR	intact	4	45	52	49	8.2
	loss	2	3	3	5	40.0
	NE	1	6	9	7	14.3
beta-catenin	nuclear	12	2	30	14	85.7
	membranous	6	36	27	42	14.3
	NE	1	4	7	5	20.0
ARID1A	intact	12	31	48	43	27.9
	loss	12	2	10	14	85.7
	NE	1	3	6	4	25.0
PTEN	intact	2	35	31	37	5.4
	loss	5	2	10	7	71.4
	het	2	3	9	5	40.0
	NE	6	6	14	12	50.0
Legend: Mut=mutated, WT=wild-type, het=heterozygous (1 core intact, 1 core loss); NE=not evaluable.						

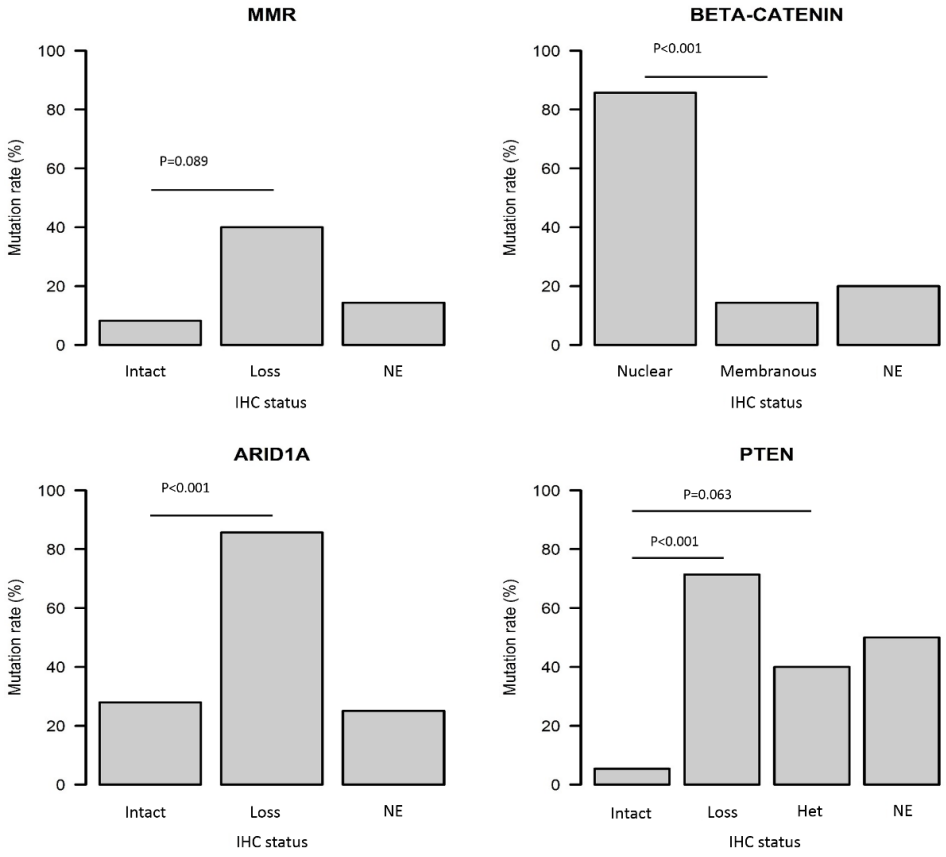

Figure 31: Mutational and immunohistochemical correlation of mismatch repair (MMR), beta-catenin, ARID1A and PTEN proteins. Figure by Dr Robert Hollis.

5.3.1 *POLE* mutations

Four (6.6%) tumours contained *POLE* mutations (1 hyper-mutated, 1 ultra-mutated) (Table 20; Figure 32). Three were missense mutations and one was a frameshift deletion. Only one pathogenic mutation occurred in the exonuclease domain. Two were novel variants not reported in the literature (p.R1675Lfs*69; p.E1062G) (Table 20). *POLE* mutations clustered solely in the *TP53*^{wt} group with none in the *TP53*^{mut} group. All four tumours had mutations in EnOC^{like} genes (100% *PTEN* and *CTNNB1*, 75% *ARID1A*, 25% *PIK3CA*), and demonstrated differences in histology and grade (Table 20). Two of these patients with *POLE* mutated tumours relapsed. One had grade 1 EnOC and was still alive 13.1 years after diagnosis, and the other with undifferentiated carcinoma died rapidly within 2 months of diagnosis. Neither of these patients' tumours were hyper- or ultra-mutated.

Table 20: *POLE* mutations in EnOC.

ID	*POLE* mutation	Exon	Mutation/ Variant	Clinical significance[a]	Hist.	Grade	Age/ Stage	Status	TMB
3874	Frame shift deletion	38	c.5024_5075del/ p.R1675Lfs*69	Unknown	Undiff	3	52, IA	Deceased	-
8462	Missense	33	c.4156C>T/ p.R1386W	VUS	EnOC	3	49, IA	Alive	Ultra
9241	Missense	9[b]	c.857C>G/ p.P286R	Path	EnOC	2	49, IIC	Alive	Hyper
9332	Missense	26	c.3185A>G/ p.E1062G	Unknown	EnOC	1	62, IIC	Relapsed and Alive	-

[a]Clinical significance as annotated on ClinVar as of July 2019.[b] Within the exonuclease domain.

Legend: VUS=variant of unknown significant; path=pathogenic; hist=histology; TMB=tumour mutation burden, hyper=hyper-mutated (>500 mutations); ultra=ultra-mutated (>3500 mutations).

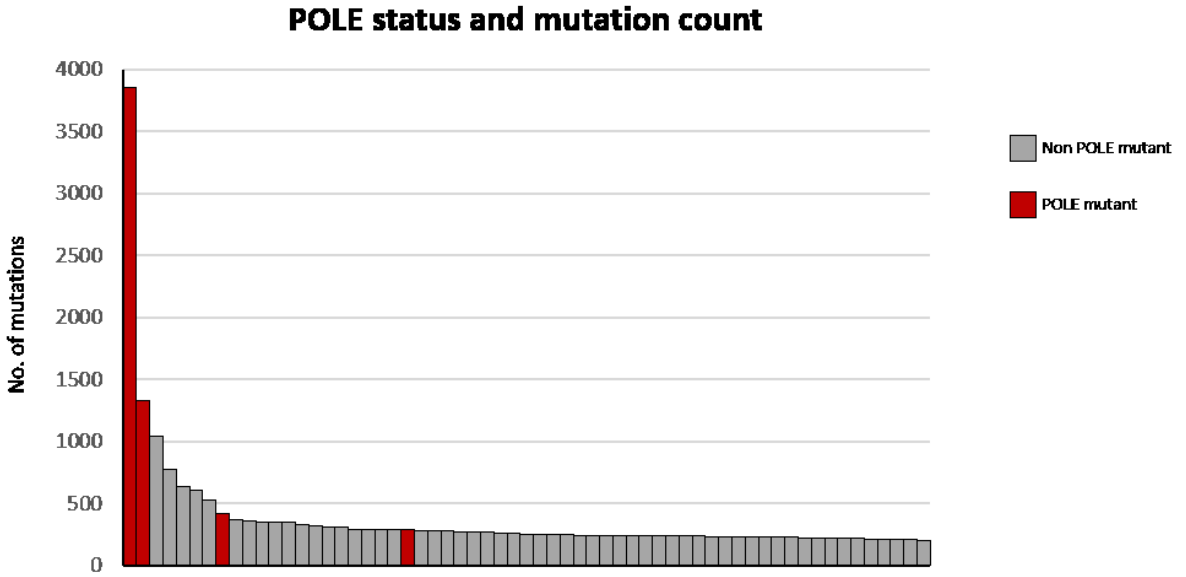

Figure 32: *POLE* mutations and mutational load. Figure by Dr John Thompson.

5.3.2 Mutations in Mismatch repair genes and *BRCA* mutations

Seven (11.5%) tumours of all grades displayed mutational inactivation in at least one of the MMR genes (Table 21). Four were low grade EnOC, and three were high grade carcinomas (Three HGS morphology, one G3 EnOC). Of nine mutations (two tumours with co-occurring MLH1 and MSH6 mutations), four were missense, whilst the rest were truncating mutations (frameshift (n=2), nonsense (n=2), splice site (n=1)). Only two mutations had their clinical significance recorded as pathogenic or likely pathogenic on ClinVar, whilst the rest were either novel (n=5) or variants of uncertain clinical significance (n=2). Four tumours had concurrent *TP53* mutations with no mutations in EnOC^{like} genes, and three were *TP53*^{wt} with EnOC^{like} mutations. The mutation rates for *MSH6*, *MLH1*, *PMS2* and *MSH2* were 6.6%, 3.3%, 3.3% and 1.6%, respectively.

Across the 125 WT1 negative tumours cohort, eight (7.3%) of 109 evaluable tumours had loss of MMR on IHC (MLH1/PMS2 (n=5), MSH2/MSH6 (n=2), MSH6 (n=1)). All occurred in tumours which were *TP53*^{wt} or displayed p53 wild-type expression on IHC. Six of these eight tumours were G1 EnOC, with the remainder occurring in high grade carcinomas (one G3 EnOC, one HGS morphology). Only two of the six evaluable tumours with mutations in the MMR genes demonstrated corresponding loss of MMR on IHC. An additional three tumours demonstrated loss of MMR protein on IHC with no mutations in MMR genes detected (MLH1/PMS2 loss (n=2), MSH6 loss (n=1)). Correlation between MMR mutational status and IHC protein expression was poor (P=0.089). Of the ten sequenced tumours which had either MMR mutations and/or loss of MMR on IHC, five (50.0%) tumours had high tumour mutation burden (hyper-mutated (n=4), ultra-mutated (n=1)), two of which were high grade carcinomas (1 G3 EnOC, 1 HGS morphology) (Table 21).

12 tumours contained *BRCA* mutations (*BRCA1* (n=5), *BRCA2* (n=7*)*). Nine were truncating mutations (frameshift (n=6), nonsense (n=2), splice site (n=1)), two were missense, and one had multiple hits (nonsense (n=2), missense (n=2)). The *BRCA* mutation variant allele frequency was greater than 0.5 for seven tumours and manually determined tumour cellularity was 50% or greater in nine tumours. Concurrent mutations were found in either EnOC^{like} genes (*ARID1A, PTEN, CTNNB1, PIK3CA*), and/or MMR genes in eight tumours (66.7%) (Table 22). Of these eight tumours, six were low grade EnOC (containing both *TP53*^{wt} and *TP53*^{mut}), and two high grade carcinomas (1 G3 EnOC, 1 HGS morphology). Four *BRCA* mutated tumours did not contain mutations in EnOC^{like} or MMR genes, and displayed classical HGS or undifferentiated morphology.

Interestingly, the only ultra-mutated tumour was diagnosed as grade 3 EnOC, and contained concurrent pathogenic *MSH2* and pathogenic *BRCA2* mutation as well as mutations in *POLE, PTEN, CTNNB1* and *PIK3CA,* but not *TP53.*

Table 21: Tumours with inactivation of mismatch repair (MMR) genes and/or loss of MMR on immunohistochemistry.

Pat ID	Age	EnOC[like]	*TP53*	MMR Gene	MMR mutation type			Variant significance[b]	MMR IHC loss	Hist.	Stage	TMB
2210	60	wt	mut	PMS2	FS del	c.896del	p.Pro299Glnfs Ter8	Unknown	NE	G2 EnOC	IA	-
4006	69	wt	mut	MSH6	Missense	c.2804C>G	p.Ser935Cys	Unknown	Intact	G2 EnOC	II	-
8315	57	like	wt	MSH6	Nonsense	c.1327G>T	p.Gly443Ter	Unknown	MSH2/ MSH6	G1 EnOC	IC	Hyper
8462	49	like	wt	MSH2	Nonsense	c.1738G>T	p.Glu580Ter	Path.	Intact	G3 EnOC	IA	Ultra
8500	52	like	wt	MLH1	Splice site	c.381-1G>C	p.X127_splice	Likely path.	MLH1/ PMS2	HGS	III	Hyper
		like	wt	MSH6	Missense	c.2911G>A	p.Gly971Arg	Unknown	intact	HGS		
9693	60	wt	mut	MLH1/	Missense	c.1153C>T	p.Arg385Cys	VUS	Intact	G2 EnOC	IIC	-
		wt	mut	MSH6	Missense	c.2511C>G	p.His837Gln	VUS	Intact			
22106	56	wt	mut	PMS2	FS del	c.896del	p.Pro299Glnfs Ter8	Unknown	Intact	HGS	IB	-
6595	61	like	wt	None	-	-	-	-	MLH1/ PMS2	G1 EnOC	IA	Hyper
7136	60	like	wt	None	-	-	-	-	MLH1/ PMS2	G1 EnOC	IV	Hyper
21860	59	like	wt	None	-	-	-	-	MSH6 loss	G3 EnOC	IC	-
8306[a]	38	like(IHC)	wt(IHC)	Not sequenced	-	-	-	-	MSH2/ MSH6	G1 EnOC	IC	NA
21982[a]	54	like(IHC)	wt(IHC)	Not sequenced	-	-	-	-	MLH1/ PMS2	G1 EnOC	IIC	NA
22143[a]	59	like(IHC)	wt(IHC)	Not sequenced	-	-	-	-	MLH1/ PMS2	G1 EnOC	IIC	NA

[a]p53 wild-type expression on IHC, EnOC[like] based on loss of protein IHC expression for either *PTEN* or *ARID1A*, or nuclear staining of beta-catenin. The rest of the EnOC[like] samples are mutational loss of *either PTEN, ARID1A, CTNNB1* or *PIK3CA* mutations.
[b]Clinical significance as annotated on ClinVar as of July 2019.
Legend: MMR=mismatch repair; mut= mutation; IHC=immunohistochemistry; Hist=histology; G=grade; TMB=tumour mutation burden; wt=wild-type; mut=mutated FS del=frameshift deletion; path=pathogenic; VUS=variant uncertain significance; EnOC=endometrioid; HGS=high grade serous morphology; Ultra=ultra-mutated (>3500 mutations); Hyper=hyper-mutated (>500 mutations); Not Seq= not sequenced.

Table 22: Clinical pathological and genomic characteristics of tumours with *BRCA* mutations.

ID	BRCA	Mutation			VAF	Cellularity (%)[b]	BRCA Var. Sig[a]	EnOC[like] profile	*TP53*	MMR mut.	MMR Var. Sig[a].	Hist.	Stage	HGS-profile
7379	1	FS del	c.2681_2682del	p.Lys894ThrfsTer8	0.72	>80	Path	wt	wt	no	-	Undiff	IIB	No
7523	2	FS insert	c.6129dup	p.Gly2044ArgfsTer5	0.34	80	Path	like	wt	no	-	G1 EnOC	IIB	No
7920	1	Missense	c.1897C>T	p.Pro633Ser	0.45	60	VUS	like	wt	no	-	G1 EnOC	IA	No
21776	2	Nonsense	c.2409T>G	p.Tyr803Ter	0.31	60	Path	like	mut	no	-	G1 EnOC	IA	No
2210	2	FS del	c.4876_4877del	p.Asn1626SerfsTer12	0.68	80	Path	wt	mut	yes	Unknown	G1 EnOC	IA	No
4006	2	Nonsense	c.37G>T	p.Glu13Ter	0.62	30	Path	wt	mut	yes	Unknown	G2 EnOC	II	No
8462	2	Missense	c.2585A>T	p.Lys862Ile	0.19	30	Unknown	like	wt	yes	Path	G3 EnOC	IA	No
8462	2	Nonsense	c.2659G>T	p.Glu887Ter	0.16	30	Unknown	like	wt	yes	Path	G3 EnOC	IA	No
8462	2	Nonsense	c.5782G>T	p.Glu1928Ter	0.23	30	Path	like	wt	yes	Path	G3 EnOC	IA	No
8462	2	Missense	c.9428T>G	p.Phe3143Cys	0.21	30	Unknown	like	wt	yes	Path	G3 EnOC	IA	No
9693	1	FS del	c.3756_3759del	p.Ser1253ArgfsTer10	0.84	60	Path	wt	mut	yes	VUS	G2 EnOC	IIC	No
22106	2	FS del	c.4876_4877del	p.Asn1626SerfsTer12	0.90	>80	Path	wt	mut	yes	Unknown	HGS	IB	No
9231	1	FS del	c.1961del	p.Lys654SerfsTer47	0.72	>80	Path	wt	mut	no	-	HGS	IIIC	Yes
9759	2	Missense	c.4436G>T	p.Ser1479Ile	0.39	20	Unknown	wt	mut	no	-	HGS	IIIC	Yes
9759	2	Missense	c.8587G>C	p.Glu2863Gln	0.12	20	Unknown	wt	mut	no	-	HGS	IIIC	Yes
21562	1	Splice site	c.80+1G>T	p.X27_splice	0.89	80	Path	wt	mut	no	-	Undiff	IV	Yes

[a]Clinical significance as annotated on ClinVar as of July 2019. [b]Manual quantification of tumour cellularity.
Legend: MMR=mismatch repair; mut= mutation; Hist=histology; G=grade; FS del=frameshift deletion; path=pathogenic; VAF= variant allele frequency; Var. Sig.= variant significance; VUS=variant uncertain significance; EnOC=endometrioid; HGS=high grade serous morphology; EnOC[like] profile=one or more mutations in *PTEN*, *ARID1A*, *CTNNB1* or *PIK3CA*. HGS-profile=*TP53* mutations with no MMR or EnOC[like] mutations.

5.3.1 Prognostic impact of *TP53, ARID1A* and *CTNNB1* mutation status

Upon univariable analysis, *TP53*^{mut} was associated with inferior DSS versus *TP53*^{wt} cases (HR for *TP53*^{wt}=0.29[0.13-0.48], P=0.004), while *ARID1A* and *CTNNB1* mutations (*ARID1A*^{mut} and *CTNNB1*^{mut}) were significantly associated with prolonged DSS (HR for *ARID1A*^{mut}=0.32[0.12-0.85] P=0.022; HR for *CTNNB1*^{mut}=0.23[0.07-0.78]; P=0.018). Collectively, EnOC^{like} cases (*PTEN*, *CTNNB1*, *ARID1A*, or *PIK3CA* mutation) had prolonged DSS versus the EnOC^{wt} group (HR for EnOC^{like} =0.32[0.14-0.73]; P=0.007). Similarly, these events were associated with significantly differential RFS (Table 23). Compared to *TP53*^{mut} cases, *TP53*^{wt} cases were more likely to be of low grade (71.8% versus 46.4%; P=0.0323), present with earlier stage disease (84.4% versus 53.6%; P=0.0206), and were more likely to undergo surgical cytoreduction to <2cm residual disease (90.3% versus 63.0%; P=0.0351) (Table 24). *TP53* mutation status was independently associated with inferior DSS and RFS upon multivariable analysis (P=0.040 and P=0.003) (Table 23, Figure 33), and this differential effect was most marked in stage II cases (DSS: HR=0.17, P=0.006, RFS: HR=0.13, P<0.001) (Figure 34).

Table 23: Univariable and multivariable survival analysis of mutations in EnOC.

		Univariable				Multivariable[a]			
Variable		**RFS**		**DSS**		**RFS**		**DSS**	
		HR(95%CI)	P	HR(95%CI)	P	HR(95%CI)	P	HR (95%CI)	P
TP53[b]	mut	ref		ref		NA		NA	
	wt	0.28 (0.13-0.63)	0.002	0.29 (0.12-0.67)	0.004	NA		NA	
***TP53* status**	mut	ref		ref		ref		ref	
	wt	0.24 (0.13-0.45)	<0.001	0.25 (0.13-0.48)	<0.001	0.25 (0.10-0.62)	0.003	0.35 (0.13-0.95)	0.040
ARID1A	mut	0.41 (0.17-0.96)	0.040	0.32 (0.12-0.85)	0.022	0.57 (0.17-1.92)	0.362	0.51 (0.13-2.02)	0.339
	wt	ref		ref		ref		ref	
CTNNB1	mut	0.29 (0.10-0.83)	0.021	0.23 (0.07-0.78)	0.018	0.38 (0.06-2.36)	0.269	0.41 (0.09-1.87)	0.247
	wt	ref		ref		ref		ref	
EnOC^{like} profile	EnOC^{like}	0.35 (0.16-0.76)	0.008	0.32 (0.14-0.73)	0.007	0.65 (0.20-2.14)	0.482	0.65 (0.17-2.52)	0.531
	EnOC^{wt}	ref		ref		ref		ref	

[a]accounting for stage, residual disease, decade of diagnosis, disease grade and age.
[b]sequenced tumours only.
Legend: HR=hazard ratio. CI=95% confidence intervals; *TP53* mutant (mut) status= tumours with a *TP53* mutation; *TP53* wild-type (wt) status= sequenced tumours which are *TP53* wild-type and non-sequenced low grade EnoC with p53 wild-type expression on IHC; NA=not applicable.

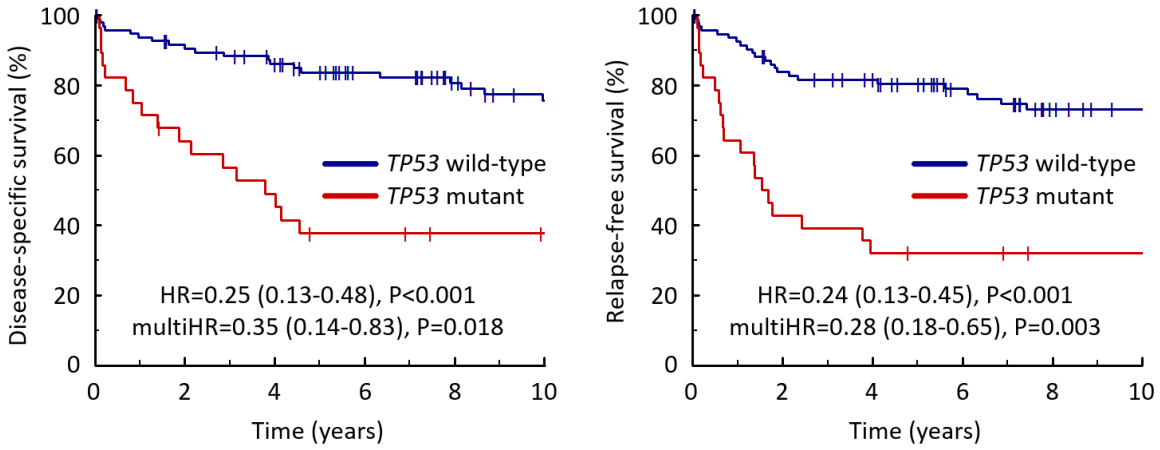

Figure 33: Disease specific survival (A) and relapse free survival(B) for *TP53* wild-type status versus *TP53* mutant status tumours.

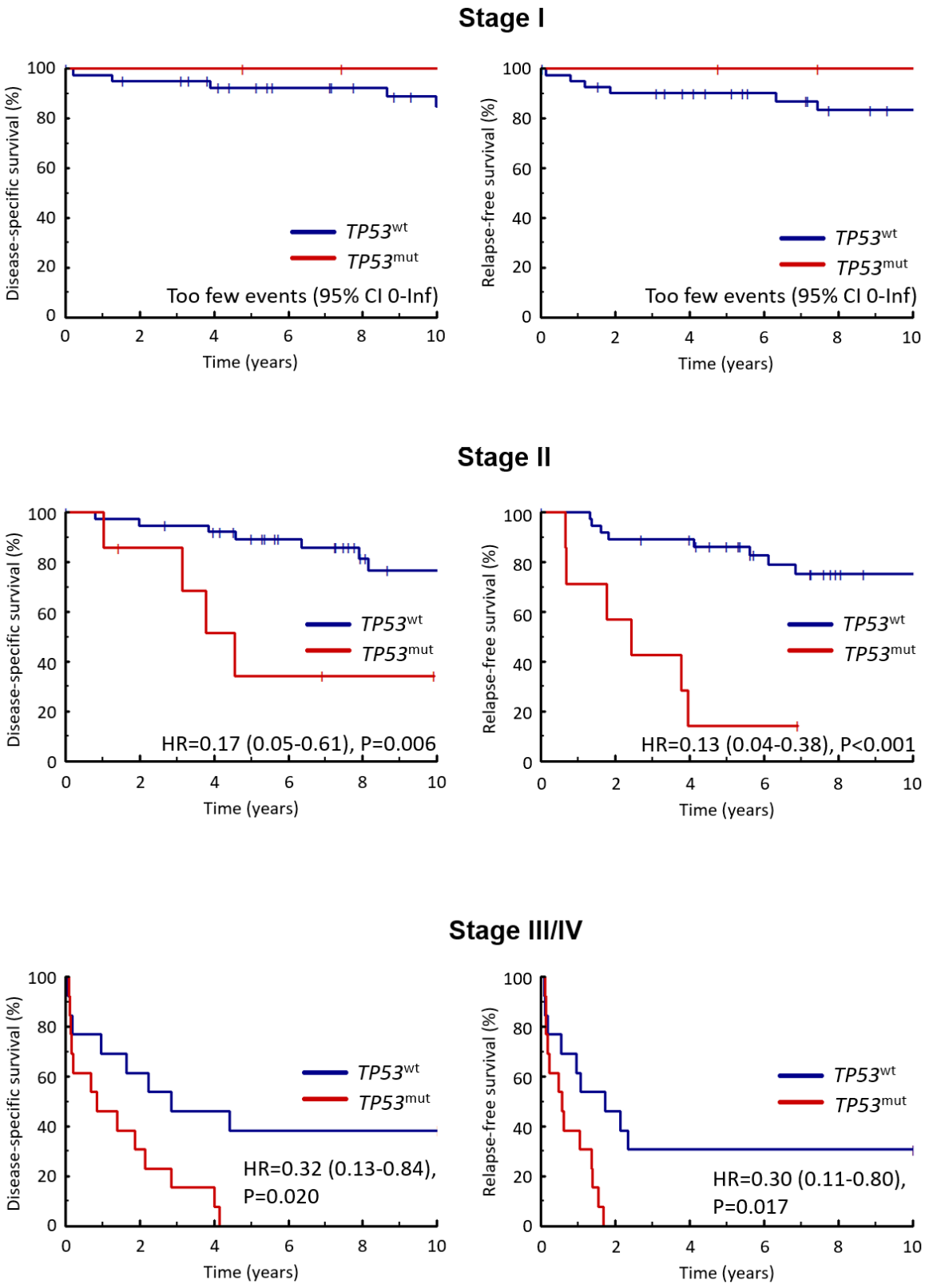

Figure 34: Disease specific survival and relapse free survival by stage in *TP53* wild-type versus *TP53* mutant status tumours.

5.4 A molecular taxonomy for classifying EnOC

A molecular taxonomy for classifying EnOC^{like} and EnOC^{wt} tumours by *TP53* and *ARID1A* mutation status, as the most common events across the cohort, was proposed. This yielded 5 subgroups: EnOC^{like}:*TP53*^{mut} (n=8; 13.1%), EnOC^{like}:*ARID1A*^{wt} (n=6; 9.8%), EnOC^{like}:*ARID1A*^{mut} (n=22; 36.1%), EnOC^{wt}: *TP53*^{mut} (n=20; 32.8%), and EnOC^{wt}:*TP53*^{wt} (n=5; 8.2%) (Figure 35). Clinical characteristics of these groups are outlined in Table 24. Molecular heterogeneity was displayed in both low grade EnOC and high grade carcinomas (Figure 35). In the 23 classical low grade EnOC (WT1 negative p53 wild-type IHC expression), 18 (78.3%) clustered in the EnOC^{like}:*TP53*^{wt} groups (*ARID1A*^{wt} (n=3), *ARID1A*^{mut} (n=15)), whilst the remaining five clustered across the other groups. Of the 12 WT1 negative p53 mutated IHC expression low grade EnOC, 11 (91.7%) clustered in the *TP53*^{mut} groups (EnOC^{like}:*TP53*^{mut} (n=4); EnOC^{wt}:*TP53*^{mut} (n=7)), whilst only one clustered in the EnOC^{like}:*ARID1A*^{wt} group. All the high grade carcinomas, which comprised eight grade 3 EnOC and three undifferentiated carcinomas, clustered across the five molecular groups. Of the 15 WT1 negative HGSOC, three (20.0%) clustered in the EnOC^{like}:*ARID1A*^{mut}, two (13.3%) in the EnOC^{like}:*TP53*^{mut} group, and ten (66.7%) in the EnOC^{wt}:*TP53*^{mut} group.

Hierarchical clustering of the Pearson correlation scores across a binary matrix of mutation status for the 100 most commonly mutated genes across the cohort resulted in a near identical stratification set to the supervised approach (Figure 36), with *ARID1A* and *TP53* status representing the most prominent stratifying mutations.

The EnOC^{like}:*ARID1A*^{mut} and EnOC^{like}:*ARID1A*^{wt} subgroups demonstrated significantly prolonged RFS compared to the EnOC^{wt}:*TP53*^{mut} group (HR=0.25 [0.10-0.66], P=0.0051 and HR=0.13 [0.02-0.99], P=0.0490; 5-year RFS 72.7% and 90.9% versus 25.0%, respectively) (Table 25, Figure 37).

DSS was significantly longer in the EnOC^{like}:*ARID1A*^{mut} cases (HR=0.24 [0.08-0.67], P=0.0063 versus EnOC^{wt}:*TP53*^{mut} group; 5-year DSS 77.0% vs 30.0%). 5-year DSS in the EnOC^{like}:*ARID1A*^{wt} group was 100% versus 30.0% in the EnOC^{wt}:*TP53*^{mut} cases , but the difference did not reach statistical (HR=0.14[0.02-1.11], P=0.0623) (Table 25).

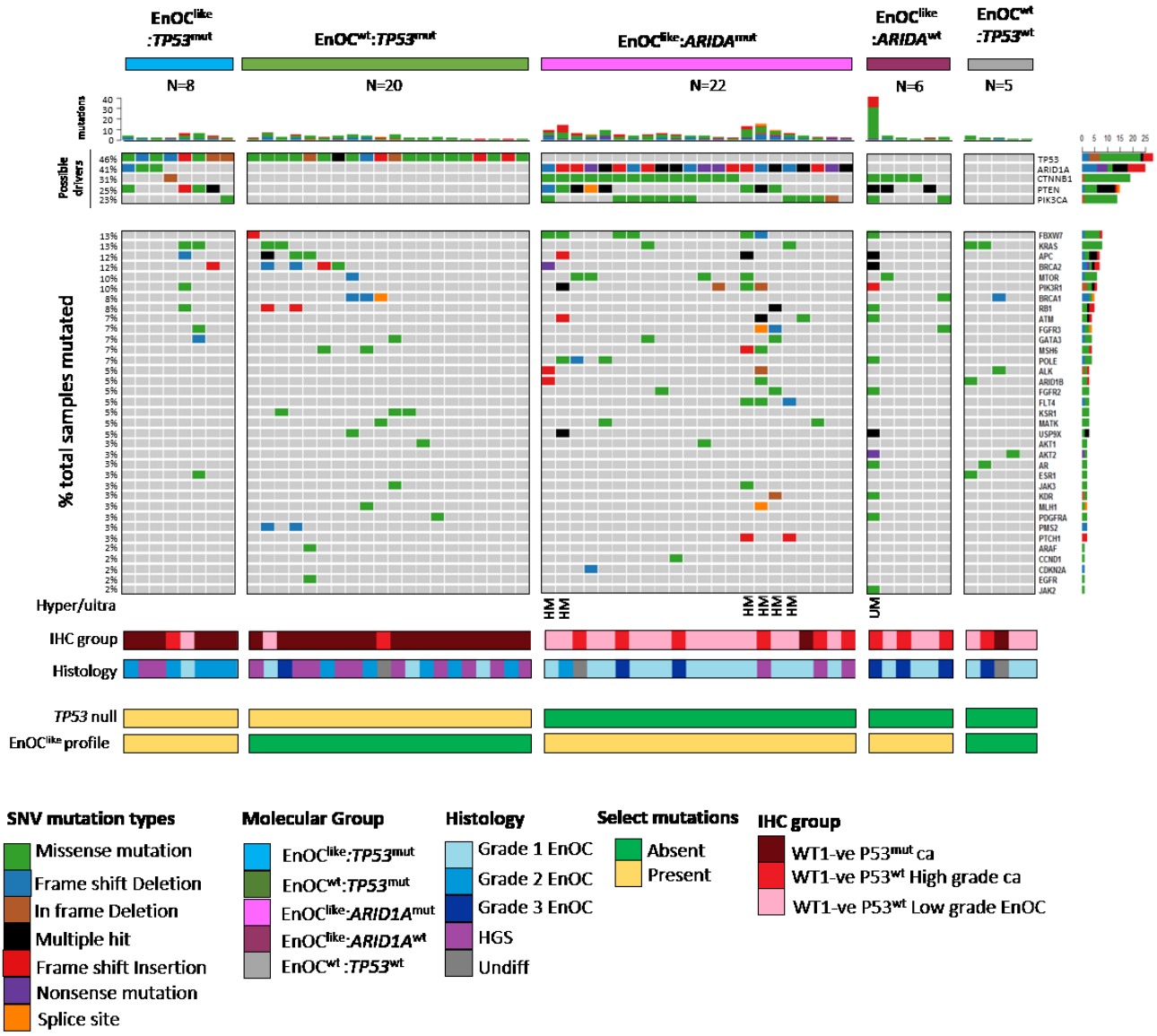

Figure 35: Oncoplot displaying single nucleotide variant frequencies identify distinct molecular groups in EnOC. Figure by Dr John Thompson.

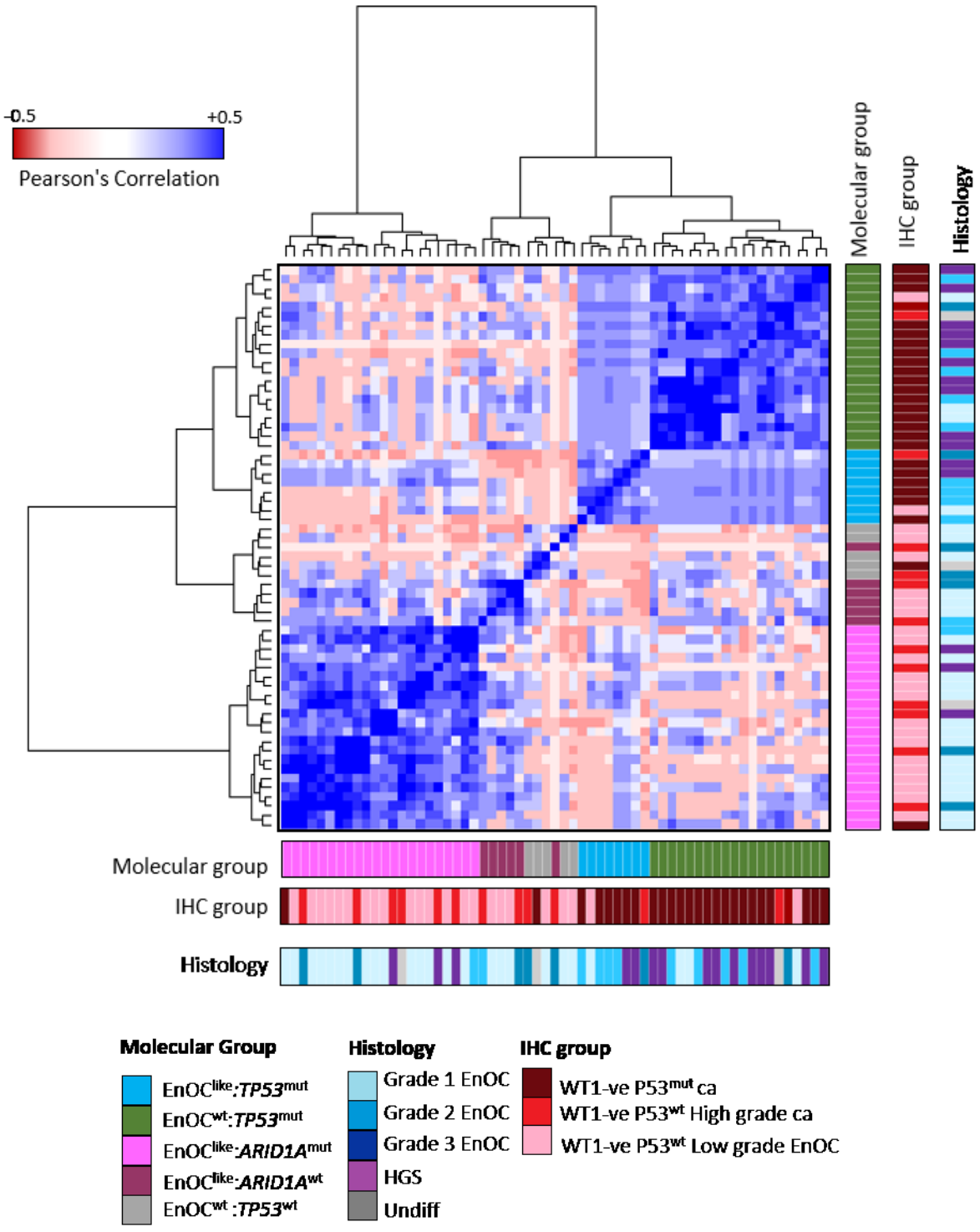

Figure 36: Plot of unsupervised hierarchical clustering of Pearson's correlation scores across the top 100 genes mutated across the whole exome dataset against the molecular groups identified in Figure 35. Figure by Dr John Thompson.

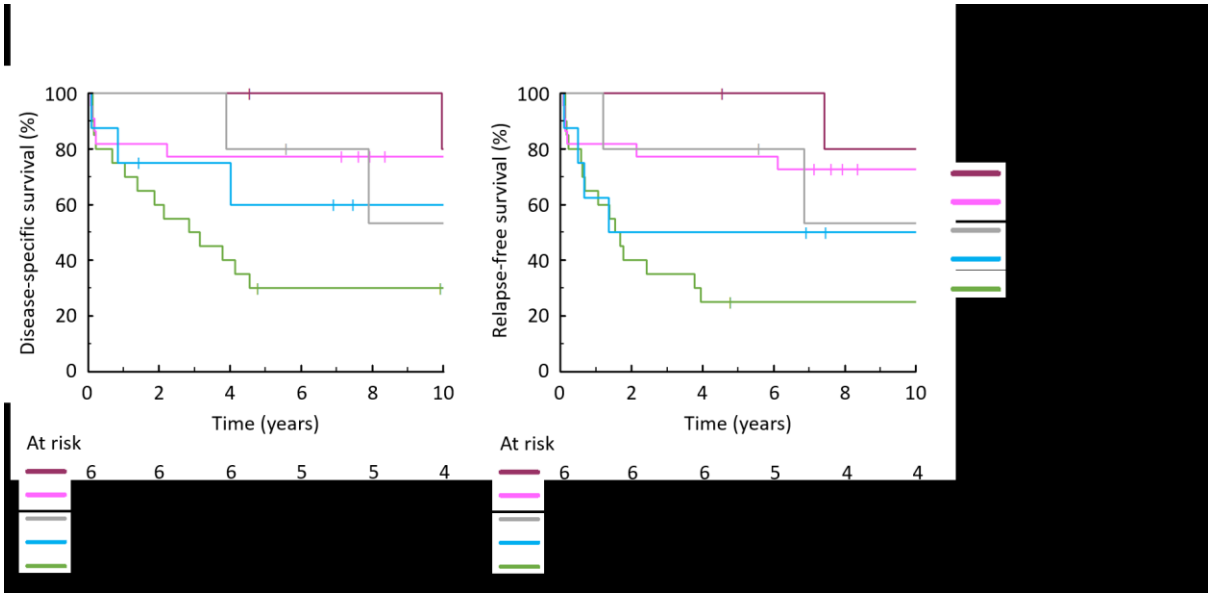

Figure 37: Kaplan Meier survival curves for disease specific survival and relapse free survival for each of the molecular groups in EnOC. Figure by Dr Robert Hollis.

Table 25: Relapse free survival and disease specific survival in the molecular groups of EnOC.

Molecular group	Relapse Free Survival				Disease Specific Survival			
	five year	ten year	HR [95% CI]	P-value	five year	ten year	HR [95% CI]	P-value
EnOC^{like}:*ARID1A*^{wt}	90.9	68.1	0.13 [0.02-0.99]	0.0490	100.0	80.0	0.14 [0.02-1.11]	0.0623
EnOC^{like}:*ARID1A*^{mut}	72.7	72.7	0.25 [0.10-0.66]	0.0051	77.0	77.0	0.24 [0.08-0.67]	0.0063
EnOC^{wt}: *TP53*^{wt}	80.0	53.3	0.36 [0.08-1.59]	0.1780	80.0	53.0	0.38 [0.09-1.66]	0.1977
EnOC^{like}:*TP53*^{mut}	50	50	0.60 [0.20-1.82]	0.3663	60.0	60.0	0.48 [0.14-1.69]	0.2551
EnOC^{wt}:*TP53*^{mut}	25.0	25.0	Ref	Ref	30.0	30.0	Ref	Ref

Table 24: Clinical characteristics of the molecular groups in EnOC.

	Molecular groups										
	EnOC^{like}: *ARID1A*^{wt} (n=6)	**%**	**EnOC^{like}: *ARID1A*^{mut} (n=22)**	**%**	**EnOC^{like}: *TP53*^{mut} (n=8)**	**%**	**EnOC^{wt}: *TP53*^{mut} (n=20)**	**%**	**EnOC^{wt}: *TP53*^{wt} (n=5)**	**%**	**P-value: *TP53*^{mut} *vs.* *TP53*^{wt}**
Age	51(37-62)		54.5(28-70)		61(41-76)		60.5(32-79)		63(37-46)		0.128
Endometrial cancer	1	16.7	5	22.7	0	0	1	5.0	1	20.0	0.0597
Endometriosis	2	33.3	8	36.4	3	37.5	3	15.0	2	40.0	0.3208
Histology											
G1 EnOC	3	50.0	15	68.2	1	12.5	3	15.0	3	60.0	0.0323
G2 EnOC	0	0	1	4.5	4	25.0	5	25.0	0	0	
G3 EnOC	3	50.0	2	9.1	1	12.5	1	5.0	1	20.0	
High grade serous	0	0	3	13.6	2	25.0	10	50.0	0	0	
Undifferentiated	0	0	1	4.5	0	0	1	5.0	1	20.0	
Year of Diagnosis											
1980s	3	50.0	5	22.7	1	12.5	1	5.0	1	20.0	0.3031
1990s	2	33.3	9	40.9	3	37.5	10	50.0	3	60.0	
2000s	0	0	7	31.8	2	25.0	6	30.0	0	0	
2010s	1	16.7	1	4.5	2	25.0	3	15.0	1	20.0	
Stage											
I	3	50.0	11	50.0	3	37.5	5	25.0	2	40.0	0.0206
II	3	50.0	5	22.7	2	25.0	5	25.0	3	60.0	
III	0	0	3	13.6	1	12.5	6	30.0	0	0	
IV	0	0	2	9.1	2	25.0	4	20.0	0	0	
UK	0	0	1	4.5	0	0	0	0	0	0	
Surgical Cytoreduction[a]											
<2cm	6	100.0	18	81.8	7	87.5	10	50.0	4	80.0	0.0351
≥2cm	0	100.0	3	13.6	1	12.5	9	45.0	1	20.0	
UK	0	0	2	9.1	0	0	1	5.0	0	0	
Received 3 cycles of platinum											0.5751
Yes	3	50.0	11	50.0	5	62.5	14	70.0	5	100.0	
No	3	50.0	11	50.0	3	37.5	6	30.0	0	0	

.[a]Classification of optimal surgical cytoreduction changed over time; some of the patients were diagnosed at a time when resection to <2cm was considered optimal.
Legend: G=grade; EnOC=endometrioid ovarian carcinoma; UK=unknown.

5.5 WT1 positive low grade endometrioid ovarian carcinomas

All six WT1 positive low grade EnOC (G1 EnOC (n=4), G2 EnOC (n=2)) cases clustered in the EnOC^{like}: *ARID1A*^{wt} group (*CTNNB1* mutation (n=5), *PTEN* mutation (n=2)) (Figure 38). All presented with early stage disease (stage I (n=5), stage II (n=1)) and were optimally surgically cyto-reduced. Ten year RFS and DSS was 100%. Five tumours were ER and PR positive (ER histoscore>200 (n=3), ER 150-200 (n=1), ER 50-100 (n=1), all 5 tumours PR 250-300). AR histoscores were mostly low (AR histoscore<100 (n=4), AR 101-150 (n=1), AR 201-250 (n=1)). No loss of MMR on IHC were observed for any tumours.

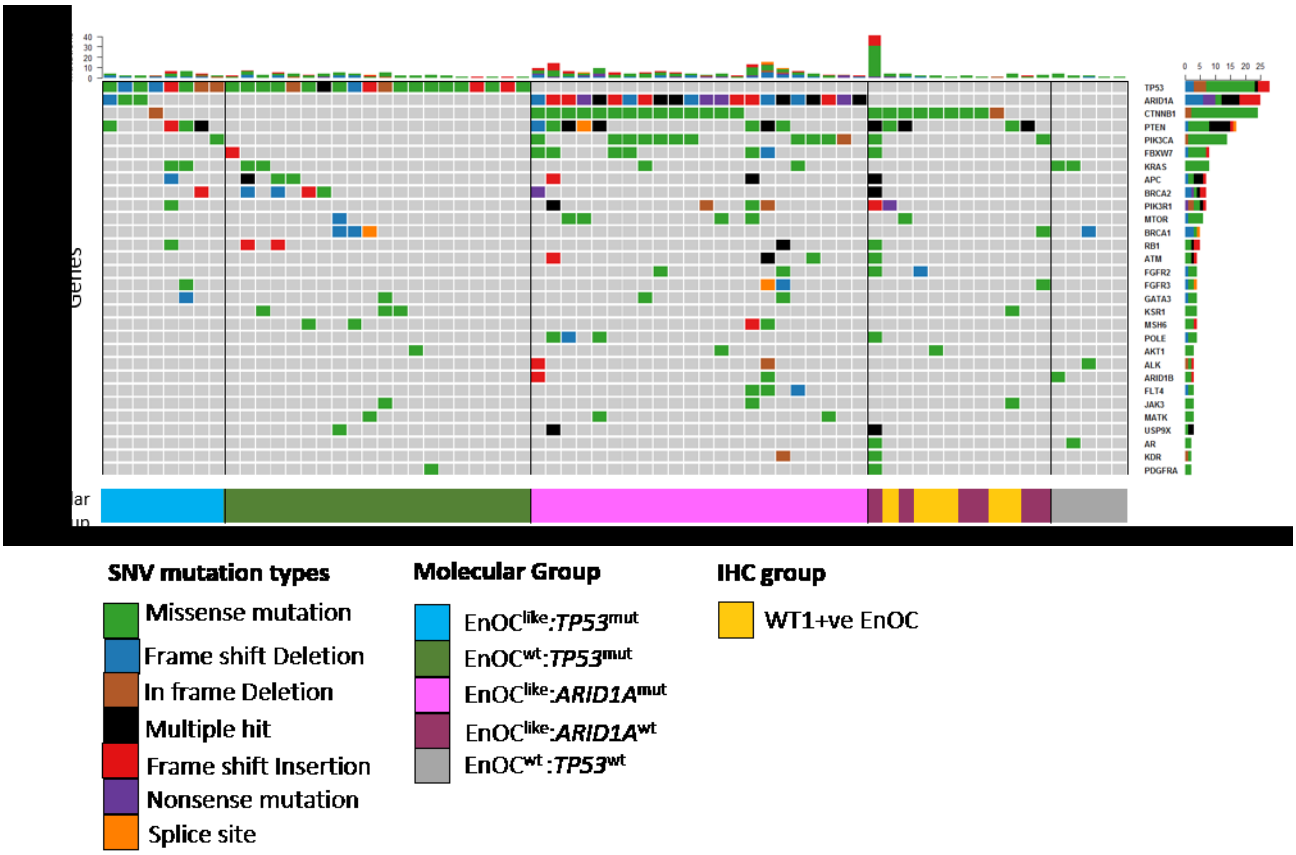

Figure 38: Oncoplot of the molecular groups identified in EnOC with all six WT1 positive low grade EnOC clustering in the EnOC^{like}:*ARID1A*^{wt} group. Figure by Dr John Thompson.

5.6 Copy number variants across molecular subgroups of EnOC

Copy number across the virtual 75 EnOC gene panel were investigated for CNVs. *TP53*^{mut} harboured greater CNVs across these genes compared to *TP53*^{wt} tumours (P<0.0001) (Figure 39). The majority of identified CNVs were copy number loss events (n=229). These included loss of *APC* (23.0%), *WT1* (21.3%), *BRCA1* (19.7%), and *TP53* (9.8%) genes across the 61 tumour samples. Copy number gain events (n=157) were most commonly detected in *PIK3CA* (19.7%) (Figure 39).

CNVs over EnOC^{like} genes (*ARID1A*, *CTNNB1*, *PTEN*, *PIK3CA*) were identified in 16 (26.2%) of cases, including eight (40.0%) of EnOC^{wt}:*TP53*^{mut} tumours (Figure 39). *APC* loss was common in the EnOC^{wt}:*TP53*^{mut} group (ten cases, 50.0%). Six tumours demonstrated copy number loss over MMR genes (*PMS2* (n=4), *MLH1* (n=2)). Four were not detected by SNV analysis, with two occurring in the EnOC^{wt}:*TP53*^{mut} groups.

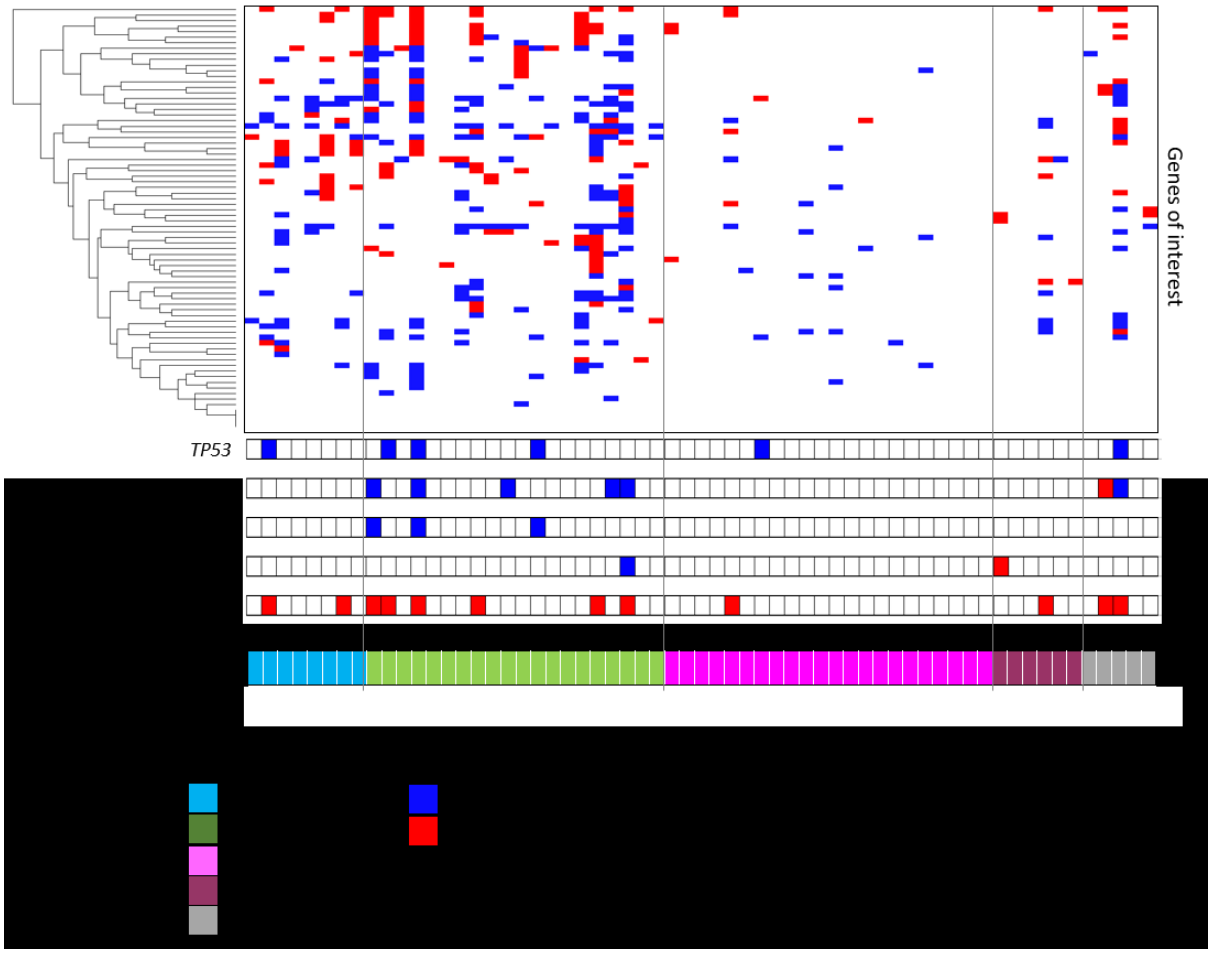

Figure 39: Plot of copy number gains and losses over the most commonly mutated genes in EnoC.
Figure by Dr John Thompson.

5.7 Hormone receptor expression in *TP53* mutant and *TP53* wild-type status tumours

In section 4, strong PR (histoscore >150), but not strong ER, was identified as an independent predictor of DSS in EnOC. The proportion of strong PR was significantly higher in the *TP53*^{wt} status group compared to *TP53*^{mut} status (P=0.003). No differences were observed between both molecular groups for ER (P=0.1096) or AR (P=0.730). Upon multivariable analysis of stage, residual disease, age, decade of diagnosis, disease grade, PR and *TP53* mutation status, strong PR remained an independent predictor of DSS (HR 0.26 (95%CI 0.08-0.81); P=0.020) but not RFS (HR 0.49 (95%CI 0.19-1.27); P=0.140), whereas *TP53* mutation status lost significance for both DSS (P=0.394) and RFS (0.104) (Table 26).

Table 26: Multivariable analysis of PR and *TP53* mutation status[a].

	Relapse Free Survival		Disease specific survival	
	HR (95% CI)	**P**	**HR (95% CI)**	**P**
PR histoscore				
>150	0.49 (0.19-1.27)	0.140	0.26 (0.08-0.81)	0.020
≤150	ref	ref	ref	ref
***TP53* status**				
mut	ref	ref	ref	ref
wt	0.43 (0.16-1.19)	0.104	0.61 (0.20-1.89)	0.394
Stage				
I	0.34 (0.11-1.04)	0.059	0.47 (0.14-1.60)	0.224
II	ref	ref	ref	ref
III	2.45 (0.76-7.87)	0.133	3.22 (0.94-11.0)	0.062
IV	5.18 (1.02-26.3)	0.047	7.41 (1.34-40.8)	0.022
Grade				
Low grade EnOC (grade 1 and 2)	ref	ref	ref	ref
High grade EnOC (grade 3)	1.20 (0.25-5.81)	0.822	1.06 (0.20-5.69)	0.944
HGS/undiff	0.71 (0.23-2.25)	0.565	1.04 (0.30-3.53)	0.954
Cytoreduction				
<2cm	0.19 (0.04-0.90)	0.036	0.18 (0.03-0.94)	0.042
2-5cm	0.74 (0.17-3.16)	0.687	0.56 (0.14-2.22)	0.405
>5cm	ref	ref	ref	ref
Decade of diagnosis				
1980s	ref	ref	ref	ref
1990s	0.43 (0.14-1.27)	0.125	0.61 (0.18-2.05)	0.421
2000s	0.29 (0.08-1.09)	0.067	0.41 (0.09-1.81)	0.241
2010s	0.22 (0.04-1.28)	0.093	0.25 (0.03-2.56)	0.245
Age	0.99 (0.95-1.03)	0.594	0.99 (0.95-1.04)	0.767

Legend: HGS=high grade serous histology; undiff=undifferentiated histology; HR=hazard ratio; CI=confidence intervals; ref=reference; [a]accounting for stage, residual disease, decade of diagnosis, disease grade and age.
TP53 mutant (mut) status= tumours with a TP53 mutation; TP53 wild-type (wt) status= sequenced tumours which are TP53 wild-type and non-sequenced low grade EnOC with p53 wild-type expression on IHC

Table 29: Multivariable analysis of PR and *TP53* mutation status[a].

	Relapse Free Survival	Disease specific survival

	HR (95% CI)	P	HR (95% CI)	P
PR histoscore				
>150	0.49 (0.19-1.27)	0.140	0.26 (0.08-0.81)	0.020
≤150	ref	ref	ref	ref
***TP53* status**				
mut	ref	ref	ref	ref
wt	0.43 (0.16-1.19)	0.104	0.61 (0.20-1.89)	0.394
Stage				
I	0.34 (0.11-1.04)	0.059	0.47 (0.14-1.60)	0.224
II	ref	ref	ref	ref
III	2.45 (0.76-7.87)	0.133	3.22 (0.94-11.0)	0.062
IV	5.18 (1.02-26.3)	0.047	7.41 (1.34-40.8)	0.022
Grade				
low grade EC (grade I/II)	ref	ref	ref	ref
high grade EC (grade III)	1.20 (0.25-5.81)	0.822	1.06 (0.20-5.69)	0.944
HGS/undiff	0.71 (0.23-2.25)	0.565	1.04 (0.30-3.53)	0.954
Surgical				
Cytoreduction				
<2cm	0.19 (0.04-0.90)	0.036	0.18 (0.03-0.94)	0.042
2-5cm	0.74 (0.17-3.16)	0.687	0.56 (0.14-2.22)	0.405
>5cm	ref	ref	ref	ref
Decade of diagnosis				
1980s	ref	ref	ref	ref
1990s	0.43 (0.14-1.27)	0.125	0.61 (0.18-2.05)	0.421
2000s	0.29 (0.08-1.09)	0.067	0.41 (0.09-1.81)	0.241
2010s	0.22 (0.04-1.28)	0.093	0.25 (0.03-2.56)	0.245
Age	0.99 (0.95-1.03)	0.594	0.99 (0.95-1.04)	0.767

Legend: HR=hazard ratio; CI=confidence intervals; ref=reference;
aaccounting for stage, residual disease, decade of diagnosis, disease grade and age.
TP53 mutant (mut) status= tumours with a TP53 mutation; TP53 wild-type (wt) status= sequenced tumours which are TP53 wild-type and non-sequenced low grade EnOC with p53 wild-type expression on IHC

5.8 Discussion

The molecular landscape of EnOC is poorly defined, particularly in high grade cases, due to under-investigation and historic misclassification of HGSOC as high grade EnOC in older studies [243, 246, 247]. WT1 negativity has emerged as an important discriminator of high grade EnOC from HGSOC, which displays morphological similarities [226, 229, 230, 244, 245]. Notably, WT1 negative undifferentiated carcinomas can co-occur with low grade EnOC as de-differentiated carcinomas, with emerging evidence of clonality between both components [258]. They are also associated with Lynch syndrome and can be misdiagnosed as high grade EnOC [263]. In order to investigate the landscape of EnOC with emphasis on high grade tumours, WES on both WT1 negative low grade EnOC and WT1 negative high grade tumours of endometrioid, high grade serous and undifferentiated histology was performed.

Overall, the frequencies of *PTEN (24.6%), ARID1A (41.0%), PIK3CA (23.0%), CTNNB1 (31.1%) and KRAS (13.1%)* mutations in my study were in line with the published literature of EnOC [220, 227, 228, 302]. These mutation frequencies did however contrast with that of Cybulska et al [381]. In the latter, *KRAS* (42%) and *PIK3CA* (39%) mutations were most prevalent in 36 pure EnOC with no concomitant endometrial carcinomas that underwent both massively parallel targeted sequencing (n=8) and whole genome sequencing (n=28). In contrast to my study, *ARID1A (19%)* and *TP53 (17%)* mutations occurred at lower frequencies. In Cybulska et al, pathology review was performed by different pathologists at different centres and methodology was not described. Furthermore, tumour grade was only reported for the eight tumours subjected to targeted massively parallel sequencing. The remaining 28 tumours (obtained from the study by Wang et al [375]) were also of unknown grade. These unknowns limit cross comparisons with my study. However, the differences in mutational frequencies observed are most likely due to the inclusion of both EnOC with concomitant endometrial carcinomas, as well as enrichment for high grade carcinomas in my study.

In Cybulska et al, formal comparison of 341 cancer-related genes was performed between pure EnOC with MSI-high and *POLE* exonuclease domain mutated EnOC removed, and HGSOC from TCGA [381]. Here, *KRAS, PIK3CA, PTEN* and *PIK3R1* were significantly more frequent in non-hyper-mutated pure EnOC compared to HGSOC, whereas *TP53* was significantly more frequent in HGSOC. When compared to EnEC from TCGA, the genes mutated were similar albeit at different frequencies. *PTEN, PIK3R1, ARID1A, KMT2D* and *CTCF* were more commonly mutated in EnEC than pure EnOC, whereas mutational frequencies of *KRAS* and *PIK3CA* were similar [381]. These differences held true when comparing pure EnOC to EnOC with synchronous endometrial carcinomas, consistent with the emerging data suggesting that EnOC with synchronous endometrial carcinomas are clonally related [236, 382]. Due to a higher proportion of grade 2 and grade 3 EnEC in the TCGA cohort, a matched analysis with grade 1 and 2 EnEC was performed. Here only *PTEN* mutations were significantly lower in EnOC compared to EnEC [381].

A formal comparison of the mutational repertoire of EnOC with those of EnEC and HGSOC TCGA was not performed in my study as only mutational frequencies of the 75 commonly mutated genes previously reported in either endometrial, ovarian or pan cancer studies were investigated. This represents a major limitation of this study. Future work should investigate the mutational frequencies and copy number variation across the whole exome rather than limiting this to a defined set of genes. Unlike the study by Cybulska et al, my study did not differentiate between pure EnOC and those with synchronous endometrial carcinomas. Although tumours with synchronous endometrial carcinomas were not systematically identified due to a reliance on pathology reports, ovarian metastases from an endometrial primary were excluded based on the Young and Scully criteria, thus limiting any inclusion of metastatic endometrial carcinomas. Given the growing body of evidence in the literature demonstrating that pure EnOC and those with synchronous endometrial carcinomas may be biologically different, an extension of this work could also include evaluating the mutational differences between these two cohorts.

In this study, *TP53* (45.9%) and *ARID1A* (41.0%) were the most commonly mutated genes. To date, the reported frequencies of *TP53* mutations in EnOC vary between 6.6% to 63% [228, 247, 375, 379,

381, 414]. This wide variation is due to the heterogeneity of these studies performed. Some studies did not perform contemporary pathology review [247, 414], whereas others performed molecular analysis in only low grade EnOC [228]. Other studies which underwent pathology review did not report on grade [375, 379, 381], thus limiting comparisons across studies. The high frequencies of *TP53* in this study is most likely explained by the enrichment of WT1 negative high grade carcinomas of endometrioid, high grade serous and undifferentiated histology. The morphological overlap of these tumours is well described with significant inter-observer variation when diagnosed based on morphology alone [229, 261]. As such, WT1 negativity, a validated IHC marker, was utilised to exclude classical WT1 positive HGSOC, as the main discriminator of these tumours. This high frequency is similarly described in Okuda et al [414]. Here, 63% of 29 historically diagnosed EnOC contained *TP53* mutations by PCR which were an independent prognostic factor.

In my study, multivariable analysis identified *TP53* mutation status as an independent negative prognostic biomarker over that of clinical prognostic variables including grade, whilst *ARID1A* and *CTNNB1* mutations were not. This finding is in line with several studies [302, 414] [324]. Of these, Parra-Herran et al is the only study that has evaluated the prognostic role of p53 IHC expression specifically in a cohort of pathology reviewed WT1 negative EnOC [302]. Here, the PROMISE algorithm [376], a surrogate of the endometrial molecular classifier, was applied and the group with p53 mutant IHC expression displayed the worst survival [302]. The molecular classifier was found to be independent of tumour grade and stage, although residual disease was not accounted for in this study.

In my study, a supervised taxonomy, incorporating *TP53* and *ARID1A* mutation status, was used to classify the WT1 negative tumour cohort. This approach identified subtypes of EnOC with differential clinical outcome, and this classification system was subsequently validated by unsupervised clustering. These data support the notion that *TP53* and *ARID1A*, as the most commonly mutated genes, are key molecular stratifiers in EnOC. *ARID1A* mutations have been found in EnOC and contiguous atypical endometriosis and postulated to be an early driver event [315, 316]. Liu et al performed a meta-analysis of 1432 patients with endometrium-related gynaecological cancers (CCOC, EnOC and EnEC), and found that negative *ARID1A* expression was associated with shorter PFS [417]. Loss of *ARID1A* protein expression on IHC has also been found to be associated with poorer prognosis in CCOC [321-323], tumours which share the common pre-cursor of endometriosis with EnOC [418]. Furthermore, Mao et al correlated progressive loss of *ARID1A* IHC expression to different stages of EnEC progression (0% complex atypical hyperplasia, 25% low grade endometrioid, and 44% high grade endometrioid)), highlighting its role in tumour progression in this closely related tumour type [325]. Collectively, these data suggest that *ARID1A* may be an important driver mutation in EnOC, and that the lack of observable survival differences between the *TP53*^{wt} cohorts, EnOC^{like}:*ARID1A*^{mut} and EnOC^{like}:*ARID1A*^{wt} may be due to the small cohort size. A larger sequencing study is thus warranted to evaluate the prognostic role of *ARID1A* mutations within the *TP53*^{wt} cohort of EnOC.

Within the *TP53*^{mut} cohort, tumours with concurrent EnOC^{like} mutations were also identified. The occurrence of these tumours have been documented by several studies [228, 243, 247, 254, 379],

however none have described their collective clinical behaviour due to the extreme rarity of these tumours. Notably, Madore et al identified two WT1 negative advanced stage high grade EnOC with concurrent aberrant p53 and β-catenin IHC staining [243]. One of these tumours gave rise to the well-characterised aggressive EnOC cell line, TOV112D. This cell line was derived from a 42 year old patient who was diagnosed with stage IIIC EnOC who died within three months of diagnosis despite cytoreductive surgery and platinum based chemotherapy. The EnOC^{like}:*TP53*^{mut} molecular group in my study thus represents the largest group of EnOC described to date which displays a molecular profile distinct to that of HGSOC, and are associated with poor prognosis. It is however acknowledged that seven percent of HGSOC in the TCGA study contained *PTEN* mutations [131]. In addition to EnOC [213], loss of *PTEN* has also been shown to be early events in the development of STICs, a known pre-cursor of HGSOC, as well as HGSOC [419-422]. The three tumours in this molecular group with concurrent *PTEN* and *TP53* mutations thus conceivably share a HGSOC molecular profile. However, all three tumours did display distinct endometrioid histology. It is thus of interest whether these tumours are genomically HGSOC with endometrioid differentiation, or *PTEN* mutated EnOC which have acquired a *TP53* mutation. Given the rarity of the EnOC^{like}*:TP53*^{mut} molecular group, a larger scale collaborative sequencing effort of EnOC tumours is warranted to characterise this molecular group further.

The EnOC^{wt}:*TP53*^{mut} group displayed the worst DSS and RFS. In particular, the *TP53*^{mut} tumours harboured greater CNV compared to the *TP53*^{wt} group, a finding which is likely underpinned by genomic instability in the context of *TP53* inactivation. Whilst some may argue that the presence of *TP53* mutations and high levels of CNVs in this cohort displays a mutational profile that is synonymous with HGSOC [423], a proportion of these cases harboured mutations in MMR genes or *KRAS*, arguing against this hypothesis. Furthermore, a proportion of these tumours displayed CNVs over EnOC^{like} genes and MMR genes, with nearly half displaying copy number loss over *APC*, a key tumour suppressor gene involved in the regulation of the Wnt/*β*-catenin pathway which is frequently disrupted in EnOC.

Furthermore, a few clinical characteristics of this group contrasts with that of HGSOC. 50% of the EnOC^{wt}:*TP53*^{mut} group presented with stage I and II disease. A similar proportion were also diagnosed as low grade EnOC. This highlights that *TP53* mutations occur in tumours which are histologically low grade EnOC, and that p53 IHC should be considered as part of routine clinical practice to assist with prognostication. The findings of low grade EnOC with p53 mutated expression on IHC are supported by several studies. In the study by Parra-herran et al, 71% of the 17 p53 mutated expression WT1 negative pathology reviewed EnOC tumours were low grade [302]. This contrasts with the study by Geyer et al where p53 mutated IHC expression were mainly found in grade 3 tumours, however no pathology review was performed in this study [254]. Here, a concurrent *KRAS* mutation was detected in the only grade 2 EnOC tumour with mutated p53 IHC expression [254], a finding which is similar to the two tumours with concurrent *KRAS* and *TP53* mutations found in the EnOC^{wt}*:TP53*^{mut} molecular group in my study. McConechy et al performed targeted exon capture of nine genes in 33 grade 1 and

2 EnOC [228]. Here, two tumours were also found to contain *TP53* mutations, one of which had no EnOC^{like} mutations, whilst the other had concurrent *ARID1A*, *PIK3CA* and *CTNNB1* mutations. Taken together, whilst the EnOC^{wt}:*TP53*^{mut} molecular group is likely to include a population of true WT1 negative HGSOC or indeed metastases from uterine high grade serous carcinomas, the mutational, copy number and clinical features discussed above may suggest a bonafide subgroup of *TP53*^{mut} EnOC associated with poor prognosis.

Only four (6.6%) tumours demonstrated *POLE* mutations. Two of these tumours were ultra- and hyper-mutated, as defined in this study, in keeping with its known association with *POLE* mutations. Notably, only one of the *POLE* mutations (1.6%) was located at the exonuclease domain (EDM) [424]. This is relevant as only *POLE* EDM have been reported to be important drivers of carcinogenesis. This frequency is numerically lower than that reported in other studies of EnOC (4.5%-10%) [302, 425, 426], and endometrial carcinomas (7%) [356]. In our study, the extremely low frequency of *POLE* EDM suggests it is not an important driver of carcinogenesis in EnOC. The *POLE* EDM cohorts in both endometrial [356] and EnOC [302, 381] have been shown to exhibit excellent prognosis, although similar conclusions were unable to be made from this study due to the small size of this cohort.

Nonetheless, all *POLE* mutated tumours in our study demonstrated differences in grade, a finding similar to that of Parra-Herran et al [221] and that of the endometrial TCGA [356]. This contrasts with that of Hoang et al which only found *POLE* mutations in grade 1 and 2 EnOC but none in grade 3 tumours [425]. Interestingly, one of the tumours in my study displayed undifferentiated histology with a deleterious frame shift *POLE* mutation. This is a novel finding as *POLE* mutations have been reported in de-differentiated/undifferentiated endometrial carcinomas, but not in ovarian undifferentiated carcinomas [427] to date. In our study, *POLE* mutations were also mutually exclusive with *TP53* mutations which is akin to other studies [302].

This study is the first to report on MMR gene mutations in EnOC. The mutational frequencies of MMR genes observed in our study (11.6%) were numerically lower than the frequency of MSI in EnOC (19.2%-29.0%) reported in systemic reviews and pooled meta-analyses [428], and was also lower than reported frequencies of around 30% for MSI observed in EnEC [356]. The frequency of loss of MMR IHC expression (7.3%) was however consistent with the literature [255, 302, 352, 361, 372]. Interestingly, mutations in MMR genes occurred in both high grade carcinomas as well as *TP53* mutated tumours. This is in contrast to Wang et al which performed whole genome sequencing of 29 EnOC [375]. Here, *TP53* mutations were confined to only MSS EnOC. These differences may be explained by the lack of MSI testing in my study which represents a major limitation. As most of the MMR gene mutations were novel, the uncertain functional consequences of these mutations may have contributed to this discrepancy. In contrast, loss of MMR IHC expression occurred across all grades and occurred exclusively in *TP53*^{wt} tumours in my study. Similarly, in the study by Parra-herran et al which evaluated the endometrial molecular classifier (PROMISE algorithm) in pathology reviewed WT1 negative EnOC, none of the tumours had concurrent loss of MMR and p53 mutant expression on IHC [302]. This suggests that dMMR in EnOC are likely to be mutually exclusive with *TP53* mutations.

In this study, concordance between mutations in MMR genes and IHC was poor. This may be due to the fact that MLH1 promoter hyper-methylation and MSI analysis was not performed [429]. In addition, over half of the discordant samples (MMR gene mutation but intact IHC expression) were missense mutations, a type of mutation which is known to have subtle effects on protein expression of MMR genes [430].

In my study, MSH6 was the most prevalent gene mutation. The predominance of MSH6 mutations in our study is similar to the study performed by Pal et al of MMR gene mutations in 1893 patients with EOC [367]. Here, 55 patients had germline mutations in MMR genes. Of these, nine pathogenic variants were detected, five of which were in the MSH6 gene with the same proportion represented by EnOC. Loss of MMR IHC expression has been demonstrated in between 7-14% of EnOC [352, 368, 370, 372, 373], which was similar to the frequency found in my study (7.3%). In contrast to other studies which found MSH2/MSH6 IHC loss to predominate [352, 372, 374], the majority in my study were comprised of MLH1/PMS2 IHC loss. This finding may be due to the presence of MLH1 promoter hypermethylation, a common somatic mechanism of dMMR, which was not performed in my study. In Bennett et al, the highest proportion of MMR IHC loss was MSH2/MSH6 in 104 unmethylated EnOC [372]. Over half of dMMR EnOC cases (n=25) in Rambau et al, which did not perform methylation analysis, were that of MSH2/MSH6 IHC loss [352]. In Chui et al, reflex testing of 48 non-serous cases also found the loss of MSH2/MSH6 and MSH6 IHC to predominate [374].

The predominance of MSH2/MSH6 loss rather than MLH1/PMS2 loss in these studies may reflect underlying LS rather than a sporadic etiology [352, 372, 374, 431], providing growing evidence that reflex dMMR testing of EnOC should be performed due to the over-representation of endometrioid histology in LS associated EOC [361, 367, 368, 371, 432]. In view of this, paired germline mutational analysis, MSI testing together with MLH1 promoter hypermethylation analysis would need to be performed as an extension of my study to investigate this further.

In my study, half of the tumours with mutations in MMR genes and/or loss of MMR expression were hyper-mutated or ultra-mutated. Wang et al showed higher frequencies of neo-antigens in MSI-high EnOC compared to MSS EnOC [375]. In a study by Xiao et al of 419 EOC, higher numbers of CD3 and CD8 positive TILs and PD-L1 intratumoural immune cells were found in tumours which were dMMR compared to those which were pMMR [361]. Similarly, Rambau et al found higher numbers of CD8 positive TILs in dMMR EnOC compared to those which were pMMR, although the authors reported poor sensitivity (64%) and specificity (81%). Taken together, these data thus suggests that dMMR EnOC represents a distinct subset of EnOC which may derive benefit from immune checkpoint inhibition in the recurrent or metastatic setting, akin to the growing body of evidence supporting its use in dMMR colorectal[433] and endometrial cancer[434]. In particular, identification of this subset of EnOC may allow access to pembrolizumab, a PD-1 inhibitor, which has garnered a pan-tumour licence by the United States Food and Drug Adminstration for all MSI-high/dMMR tumours [435].

Interestingly, *BRCA* mutations in a fifth of our study cohort, most of which were pathogenic, were detected in my study. The *BRCA* variant allele frequency was greater than 0.5 for nearly two thirds of

these tumours thus suggesting mutation of both *BRCA* alleles and that the majority of these mutations are driver mutations. Of these, concurrent mutations in EnOC^{like} and/or MMR genes in two thirds of these tumours across all grades and in both *TP53*^{mut} and *TP53*^{wt} tumours were found. Only three *BRCA*1/2 mutated tumours displayed a genomic and phenotypic profile of HGSOC. In particular, the only ultra-mutated *TP53*^{wt} tumour had both concurrent pathogenic *BRCA*2/MSH2 mutations as well as mutations in common genes associated with EnOC. This is similar to the findings in Teer et al which performed WES on six EnOC and further panel based sequencing on another 14 EnOC [379]. In both cohorts, five EnOC contained concurrent *BRCA* 2 mutations and other mutations in EnOC^{like} genes. Similarly, in Wang et al, 12% of EnOC (grade unspecified) which underwent WGS clustered in the molecular cohort associated with HRR deficiency [375]. Together, this data suggest that a subset of EnOC, including that of low grade tumours, contain mutations in HRR genes, and may not be solely confined to HGSOC [131]. This novel finding raises the hypothesis that a proportion of EnOC, including that of low grade EnOC, may also derive benefit from PARP inhibitors.

As discussed in chapter 1.5.4 and 1.5.8, the molecular profile of high grade EnOC is not well-defined as pathological definitions have evolved and many historically diagnosed high grade EnOC are now thought to be HGSOC. The unique aspect of this molecular analysis performed in my study lies in the fact that WT1 negative high grade carcinomas of endometrioid, high grade serous and undifferentiated histology were included. In chapter 1, the challenges of distinguishing high grade EnOC from HGSOC on the basis of morphology alone are discussed. The use of WT1 as an important and useful discriminator between the two subtypes is well described. In this study, all WT1 positive tumours were excluded, most of which were classical HGSOC (WT1 positive, p53 aberrant expression). Large ovarian carcinoma re-classification studies have found that over 95% of HGSOC are WT1 positive, and a combination of WT1 positivity and p53 aberrant expression is highly specific with 91.7% of these tumours displaying this combination compared to <1% in EnOC [229, 253]. As such, it is thus unlikely that any EnOC with that IHC profile (WT1 positive, p53 aberrant) were excluded. We did not rely on morphology alone to diagnose high grade EnOC and instead, relied solely on WT1 negativity, as the main inclusion criteria for high grade carcinomas of both histologies. Undifferentiated ovarian carcinomas are extremely rare entities which can be associated with low grade EnOC as de-differentiated carcinomas, with evidence of clonality between both components [257, 258]. This supports the inclusion of these carcinomas as a spectrum of EnOC in our WES cohort.

In my study, high grade EnOC and undifferentiated carcinomas display substantial molecular heterogeneity with no predilection for one molecular group. A third of the WT1 negative tumours with high grade serous morphology displayed an EnOC^{like} genotype, of which 20% were in the EnOC^{like}:*ARID1A*^{mut} group, a finding which would not have otherwise been elicited if WES was only performed on high grade EnOC diagnosed as per WHO classification. Furthermore, the molecular heterogeneity of the high grade EnOC diagnosed in our study is akin to grade 3 EnEC which did not segregate in one molecular group, but were represented in all four molecular groups of the endometrial TCGA, each with vastly different prognosis [436]. As an extension of the endometrial TCGA study, pathology consensus was evaluated in the 75 grade 3 EnEC tumours [436]. Here, inter-observer

agreement was highest in the copy number low cohort, and lowest in the copy number high cohort. In addition, of the six cases diagnosed by both expert pathologists as serous carcinomas, only two displayed the serous genotype (*TP53* mutations without *ARID1A* and/or *PTEN* mutations), whilst the remaining displayed an endometrioid genotype, findings which mirror those of my study. Similarly in EnOC, there exists some variation on what constitutes high grade EnOC diagnosed by modern criteria. For example, in Lim et al, only tumours with either confirmatory endometrioid features or contained histologically identical components to that of low grade EnEC were diagnosed as EnOC [226]. Only three percent were diagnosed as high grade EnOC and this diagnosis was made without the use of IHC. In Assem et al, most grade 3 EnOC which changed category from HGSOC following the use of WT1 and p53 IHC did not contain CEFs [224]. In a large scale pathological re-classification study of EOC based on 8 IHC markers, the two largest reclassified groups were EnOC to HGSOC (n=29), and HGSOC to EnOC (n=8). These tumours underwent targeted sequencing of 28 genes implicated in ovarian cancer. An EnOC-like mutational profile was defined as at least one mutation in *CTNNB1*, *PIK3CA*, *ARID1A*, *KRAS* or *PTEN* without a *TP53* mutation. A HGSOC-like profile was the presence of a *TP53* mutation without any EnOC-like mutations. The sequencing data confirmed the pathological reclassification in 20 cases (16 EnOC to HGSOC, and four EnOC to HGSOC), however disproved five cases of EnOC to HGSOC. Taken together, these data is in line with my study which strongly supports the use of sequencing to help differentiate between WT1 negative high grade carcinomas of endometrioid, high grade serous and undifferentiated histology.

During the pathology review process, six WT1 positive tumours with histological appearances of low grade EnOC were identified. I hypothesised that these were either true WT1 positive low grade EnOC, or LGSOC with pseudo-endometrioid appearances. Interestingly, all of them clustered in the EnOC^{like}:*ARID1A*^{wt} group, thus confirming them to be genomically EnOC. This has immediate clinical application as it provides pathological confidence to the existence of true WT1 positive low grade EnOC that are associated with excellent prognosis. This may also suggest that a subset of EnOC may arise from the ovarian surface epithelium (WT1 positive) rather than ectopic endometrial tissue (WT1 negative) [252]. This is in keeping with the study performed by Stewart et al of 41 grade 1 and 2 EnOC in which 20% were positive for WT1 and demonstrated a negative correlation with endometriosis [218].

Finally, a subset of tumours with CNVs over EnOC^{like} genes in the EnOC^{wt} groups, and CNVs over the *TP53* gene in the *TP53*^{wt} groups were identified. This is akin to the study performed by Teer et al [379]. Here, one of six low grade EnOC tumours which underwent WES was found to have no mutations in common cancer genes but had the most number of CNVs. This tumour had amplification over driver genes (PIK3R1, MET, ALK and NOTCH2) and deletion of PMS2. It can therefore be hypothesised that employing copy number analysis may help refine molecular stratification of the SNV defined molecular groups of EnOC.

In chapter 4, strong PR was found to be associated with DSS independent of age, stage, residual disease, and year of diagnosis. In this chapter, the relationship between strong and weak PR, and *TP53* mutation status was investigated. Here, the degree of PR expression was associated with *TP53* mutation status with higher frequencies of strong PR found in the *TP53*^{wt} cohort compared to the

TP53^{mut} cohort. Studies in breast cancer have specifically reported this inverse relationship [437]. PR has been shown to be regulated by ER in ovarian cancer cells and mediates the protective effect of progesterone on cancer cell invasion and metastases [438, 439]. Loss of PR expression has also been associated with increasing grade in EOC which influences survival [440]. The relationship between strong PR and *TP53* is likely a reflection of this as the majority of *TP53* mutant tumours in our study were of high grade. This is also in keeping with EnEC, in which loss of PR expression is associated with poor prognosis [441, 442]. Lower expression levels of PR have also been found in metastases compared to matched primary tumours in several studies of EnEC [442].

In this chapter, *TP53* mutations were independently associated with DSS over clinical variables. Interestingly, when accounting for *TP53* mutation status and PR expression in a multivariable analysis which importantly also accounted for grade, only strong PR expression, but not *TP53* mutation status, remained independently associated with DSS. These findings draw striking parallels with one prospective multicentre study in endometrial carcinomas [441]. It found that ER and PR negativity influenced survival independent of tumour grade in lymph node negative EnEC. Notably, p53 IHC status lost significance in the multivariable model, whilst ER and PR negativity retained statistical significance. Similar to my study, the authors argue that ER and PR status was a stronger predictor of lymph node metastases and survival then the use of p53 IHC expression. This highlights strong PR expression to be a powerful prognostic biomarker in EnOC which is independent of grade, and should be considered for routine testing in clinical practice to inform prognosis and the need for adjuvant treatments.

5.9 Study Limitations

The major limitations of this study include the large number of irretrievable tumours samples which may have contribute to selection bias. Only a third of reviewed cases had available slide series for pathology review, with the remaining tumours reviewed from a single tumour block. This would have posed limitations in systematically identifying the presence of endometrioisis as well as pathology review of the endometrial tumours in cases with reported synchronous primaries.

Both SNV and CNV analysis were performed across a targeted 75 gene panel rather than across the whole exome which may have also contributed to selection bias in the molecular stratification of EnOC in my study. However, despite this, the frequencies of commonly mutated genes in EnOC in this study were largely similar to that of the literature. In addition, the molecular groups obtained through employing both supervised and unsupervised clustering methods, suggests robust exome data obtained from FFPE tumour samples.

Subsequent to this study, the methodology was further refined through repeating the SNV and copy number analysis across the whole exome rather than confining it to the 75 gene panel. As outlined in section 2.6, unsupervised analysis was performed across the top 100 differentially mutated genes within the total tumour dataset and represented as a binary matrix. Samples were clustered by Euclidian and Ward methods based on the overall Pearson correlation score of these binary signatures. In contrast to this study, supervised mutational analysis was performed using the most differentially mutated genes across the sequenced samples identified from the unsupervised clustering analysis. Through this

methodology, the same molecular cohorts were identified suggesting that 75 gene panel used in this study were representative as the major driver mutations in EnOC.

Another major bias in our study was the methodology in which sequenced tumours were selected. WES was performed on all WT1 negative tumours with p53 mutated IHC expression as well as all p53 wild-type expression high grade carcinomas of endometrioid, high grade serous and undifferentiated histology. However, due to budget constraints, only a randomly selected proportion of WT1 negative p53 wild-type expression low grade EnOC underwent WES, as it was hypothesised that these tumours were pathologically homogenous and were thus likely to be similarly so at a molecular level. However this methodology lends itself to selection bias and the mutational frequencies reported in my study are thus less representative of EnOC due to the exclusion of the remaining classical low grade EnOC. Since this study, Professor Gourley's laboratory group has since proceeded with performing WES on the remaining 64 WT1 negative p53 wild-type expression low grade EnOC, following which an unbiased comprehensive molecular profiling of EnOC will be performed, molecular subgroups identified and correlated with clinical outcome (Hollis et al, unpublished and awaiting peer review).

It is notable that I did not apply the PROMISE algorithm to my study cohort. As discussed in chapter 1, the PROMISE algorithm was developed and validated as a molecular classifier in endometrial carcinomas. Parra Herran et al was the first study to apply the PROMISE algorithm to a cohort of WT1 negative EnOC and found the molecular classifier to correlate with disease free survival in a multivariate analysis independent of grade and stage. However, akin to my study (10%), the *POLE* mutated and MMR abnormal cohorts only comprised 18% with the majority of EnOC comprised of the p53 wild-type cohort (58%) [302]. The low frequencies of the *POLE* and MMR abnormal cohorts observed thus suggests they are not key drivers within EnOC. Furthermore, as over half of the cohort in the study by Parra-Herran had no specific molecular profile, it was therefore critical to investigate if there were other molecular drivers within EnOC which could provide additional prognostic granularity through an unbiased approach. However, it is acknowledged that applying the PROMISE algorithm to my study cohort in addition may have allowed for more direct comparisons to be made in this regard.

5.10 Conclusion

In this study, WES was performed on 61 WT1 negative EnOC with particular emphasis on high grade carcinomas. The key findings are summarised as follows:

- *TP53* and *ARID1A* mutations were the most commonly mutated genes and stratified EnOC into molecular subgroups with differential clinical outcome.
- *POLE* and MMR gene mutations exist at low frequency in EnOC.
- CNVs in EnOC may be used to refine molecular classification in SNV subgroups of EnOC.
- EnOC with $TP53^{mut}$ are associated with poor prognosis independent of age, stage, residual disease and decade of diagnosis.
- EnOC with $TP53^{mut}$ have lower levels of PR expression compared to tumours which are $TP53^{wt}$.
- Strong PR expression (histoscore >150) defines a cohort of EnOC with superior prognosis independent of $TP53^{mut}$ status.

- WT1 positive low grade EnOC are genomically EnOC and demonstrate good prognosis.
- WES of the remaining un-sequenced classical low grade EnOC (WT1 negative p53 wild-type expression) cohort is warranted to validate these findings.

6. Final Discussion

6.1 Discussion

To my knowledge, this is the largest study investigating the clinical, pathological and molecular correlation in a cohort of contemporary pathology reviewed EnOC. Here, the key findings and the potential therapeutic implications of this study are summarised.

Comprehensive pathology review of 271 tumours historically diagnosed as EnOC utilising WT1 IHC was performed. All WT1 positive tumours were excluded leaving a cohort of 125 WT1 negative EnOC of all grades. A consistent approach was maintained throughout this study, utilising WT1 and p53 IHC on the corresponding whole tissue section during pathology review of every chemotherapy naïve tumour rather than a TMA, minimising the impact of tumour heterogeneity or fixation defects. The same corresponding tissue section was used for DNA extraction and WES. This is in contrast to other studies which did not use the corresponding pathology reviewed slide for WES [379], performed sequencing on EnOC of unknown grade [375, 381], included post chemotherapy samples in the analysis [375], or utilised different sequencing methods in the same analysis [379, 381].

A unique aspect of this study is the inclusion of high grade carcinomas of high grade serous, undifferentiated and endometrioid morphology as they display significant inter-observer variation. The use of WT1 negativity has been shown to be an important and useful discriminator. As discussed in chapter 1, over 95% of HGSOC are WT1 positive, and a combination of WT1 positivity and p53 aberrant expression is highly specific with 91.7% of cases displaying this classical IHC profile, whereas up to 90-96% of EnOC are WT1 negative [229, 253]. Studies have demonstrated that the use of WT1 IHC, which have resulted in a change of histotype assignment, most commonly occurs from EnOC to HGSOC. Kobel et al performed a large re-classification exercise of 1626 tumours utilising a panel of IHC markers including WT1 [229]. In this study, the most common misclassification was between EnOC

and HGSOC (29 of 72 misclassified cases), with fewer (n=8) cases reclassified from HGSOC to EnOC. Targeted panel sequencing was performed on both these cohorts and confirmed the reclassification in the 80% of cases but refuted it in 20% suggesting additional value to mutational analysis in cases with discordant histology and IHC. This further supports my rationale for the inclusion of WT1 negative tumours of high grade serous morphology in this study.

In chapter 3, both classical low grade EnOC (WT1 negative, p53 wild-type IHC expression) as well as the whole WT1 negative cohort were characterised clinically. Consistent with the literature, patients with early stage EnOC in this study had a good prognosis, whereas those with advanced stage classical low grade EnOC had a prognosis superior to that of advanced stage HGSOC. I also reported that relapses beyond five years are common in EnOC which may influence the duration of follow up as these patients are often discharged from oncology after five years. Furthermore, these tumours commonly relapse as a solitary pelvic mass, thus raising the hypothesis as to whether adjuvant radiotherapy may have a role in the management of these tumours akin to emerging data of benefit in CCOC.

In chapter 4, the majority of EnOC display high ER and PR expression as determined by weighted histoscores, whilst most tumours display low AR expression. Only a PR histoscore of greater than 150, but not ER or AR, was independently associated with prognosis. I also reported on a series of patients with EnOC which received endocrine therapy and derived prolonged benefit. This adds to the small body of published data demonstrating these tumours to be hormone sensitive and that endocrine therapy should be readily used in its management. It also raises the hypothesis of whether there is a role of endocrine therapy in the adjuvant setting for patients with EnOC.

WES on 61 tumours of this cohort was performed. This was comprised of three WT1 negative IHC cohorts: i) all p53 wild-type expression high grade carcinomas, ii) all p53-mutant expression low grade and high grade carcinomas, and iii) a random selection of p53 wild -type expression low grade EnOC. I find that EnOC is molecularly heterogeneous and is stratified by *ARID1A* and *TP53* as the predominant mutations, with each group displaying differential clinical outcomes. A subset of both low and high grade EnOC with concurrent *BRCA* mutations was also identified, suggesting that these tumours may derive benefit from PARP inhibitors. Of particular interest was the EnOC^{wt}: *TP53*^{mut} cohort which initially appear genomically akin to HGSOC; however the presence of CNVs over EnOC^{like} genes, concurrent mutations in MMR genes, with half the cohort presenting as early stage disease with endometrioid histology, suggests that this molecular subgroup is unique and warrants further investigation.

The high grade tumours in this study were found to be genomically heterogeneous, a finding akin to that of grade 3 EnEC [436]. In particular, a third of the WT1 negative tumours with high grade serous histology displayed an EnOC^{like} mutational profile, a finding which would not have been elicited if only grade 3 tumours of endometrioid morphology were solely investigated. This study therefore highlights the need to apply molecular stratification strategies to refine pathological diagnoses particularly in WT1 negative high grade carcinomas of endometrioid, serous and undifferentiated morphology akin to its emerging role in endometrial carcinomas [378, 436].

In chapter 1.5.2, the FIGO grading system used in EnOC is described, one which is extrapolated from EnEC due to shared morphology. Briefly, this is a three tier architectural grading system based on the proportion of solid growth (grade 1 <5%, grade 2 5%-50%, and grade 3 >50%). The presence of nuclear atypia in an otherwise architecturally low grade tumour increases the grade by one level.

In EnEC, there is a growing body of evidence which casts doubt on the value and reproducibility of grade 2 tumours [223, 225, 443-445]. These studies have proposed a binary grading system and demonstrated that grade 1 and 2 tumours can be combined as low grade EnEC. The main consideration of grading EnEC is to identify grade 3 EnEC in view of its adverse effect on prognosis and not an attempt to differentiate grade 1 or 2 tumours.

A similar story is unfolding in EnOC and the prognostic value of the FIGO grading system has been questioned by several retrospective studies. As described in chapter 1.5, Parra-Herran et al found the Silverberg grading system to provide superior prognostication to the FIGO grading system with a two-tier grading system favoured in EnOC [221]. In this study, the survival of patients with FIGO grade 1 and 2 tumours overlap for the first five years however grade 2 tumours approach survival of grade 3 tumours beyond five years [221]. This contrasts with that of Assem et al in which no clinical or survival differences were observed between FIGO grade 2 and 3 tumours and the authors argue that these 2 groups of tumours could be grouped together [224]. These findings mirror that of my study in which no differences were observed in DSS or RFS between grade 2 and 3 tumours. Taken together, these studies suggest that like EnEC, there exists substantial inter-observer variation in the determination of grade 2 EnOC.

In my study, both the presence of *TP53* mutations, and a PR histoscore of more than 150 (strong PR), were both independent predictors of prognosis over common clinical variables of prognosis. When accounting for both variables in a multivariable analysis which also included grade, only strong PR remained an independent predictor of prognosis whereas *TP53* mutation status lost significance. In particular, patients with stage II disease with strong PR demonstrate a five year DSS of over 90%. No differences were observed between the strong and weak PR groups in the proportion of patients receiving adjuvant platinum based chemotherapy. It can therefore be hypothesised that strong PR expression could be used to identify patients with early stage EnOC who can avoid adjuvant chemotherapy even in stage II disease.

In order for a pathological grading system to be successful, it needs to be reproducible, practical and provide clinically relevant prognostic information. In accordance with EOC being five distinct biologically and molecularly diverse histological subtypes, the grading system is unique to each. For example, although CCOC are pathologically well differentiated, they are all considered high grade due to its aggressive biology with stage being the main determinant of prognosis [129]. Similarly, as discussed in section 1.4, a revised two-tier Silverberg grading system which accounts for nuclear atypia and number of mitoses was proposed in 2004 by Malpica et al for serous ovarian carcinomas [157]. This has subsequently been proven to be highly reproducible with demonstrable differences in the molecular profile and clinical behaviour between HGSOC and LGSOC. Unlike determining grade in EnOC, the

bimodal PR expression exhibited in my study lends itself to being easily reproducible and can be readily implemented into clinical practice. This study has demonstrated that PR expression is prognostic even when accounting for grade and stage in EnOC, a finding which should be validated in a larger study with the aim of utilising this biomarker to guide decisions regarding adjuvant chemotherapy in early stage disease.

I have explored this study's limitations in the individual chapters. The major limitations relate to the high attrition rate with tumour retrieval, and due to constraints in resources, the inherent selection bias in selecting tumours for WES, and the evaluation of a 75 gene panel rather than the whole exome gene panel which pose limitations in the interpretation of mutation frequencies within this study cohort.

Nonetheless, this study has demonstrated that EnOC is a heterogeneous disease comprising distinct molecular and hormone receptor subgroups with differential clinical outcome. *TP53* mutations (or p53 mutated expression on IHC) and low PR expression (histoscore <150) have been shown in this study to be independent poor prognostic factors. Subsequent work should focus on the development of molecularly targeted agents for these subgroups of EnOC which display the poorest prognosis. In particular, this study raises the hypothesis that WES should be utilised to differentiate WT1 negative high grade carcinomas of serous, endometrioid and undifferentiated morphology which display molecular heterogeneity. In particular, the EnOC^{wt}:*TP53*^{mut} tumours identified in this study represent a group of poor prognosis which display clinical and molecular features which suggest that they are biologically distinct from HGSOC. Worldwide collaborative efforts are required to investigate this rare subgroup further. Further work should include WES of the remaining classical low grade EnOC and unsupervised clustering across the whole exome to investigate the molecular landscape in an unselected population of EnOC in order to validate the molecular and hormone receptor subgroups identified in this study.

7. Appendices

7.1 Appendix A

Whole Exome Sequencing Report

Report by: Richard Clark on 04/12/2017

Number of Samples: 72

1. Summary of Sequencing Protocol

Quality Control

DNA samples extracted from Formalin-Fixed Paraffin-Embedded (FFPE) tissue were provided by the Investigator. DNA was quantified using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific Inc, #Q32866) and the Qubit DNA BR assay kit (#Q32853).

Library Preparation

Libraries were prepared from each DNA sample using the TruSeq Exome Library Prep kit (#FC-150-1002) according to the provided protocol using modifications for working with FFPE sourced material.

200ng of DNA was end-repaired to remove 3' and 5' overhangs, and fragment length was optimised using sample purification beads. A single 'A' nucleotide was added to the 3' ends of the blunt fragments to prevent them from ligating to another during the subsequent adapter ligation reaction, and a corresponding single 'T' nucleotide on the 3' end of the adapter provided a complementary overhang for ligating the adapter to the fragment. Multiple indexing adapters were then ligated to the ends of the ds cDNA to prepare them for hybridisation onto a flow cell, before 12 cycles of PCR were used to selectively enrich those DNA fragments that had adapter molecules on both ends and amplify the amount of DNA in the library suitable for sequencing. Libraries were quantified using the Qubit 2.0 Fluorometer and the Qubit DNA HS assay (#Q32854) and the size distribution of fragments was assessed using the Agilent Bioanalyser with the DNA HS Kit (#5067-4626).

Whole Genome (WG) DNA libraries containing unique indexes were combined in pools of 6, and then target regions of the DNA were bound with capture probes. Streptavidin Magnetic Beads were then used to capture probes hybridised to the targeted regions of interest and a series of washes removed nonspecific binding from the beads. This process was repeated to ensure high specificity of the captured regions. Captured enriched library was then purified before 8 cycles of PCR amplification and a final purification step to remove unwanted products.

Library QC

Exome-captured sequencing library pools were quantified using the Qubit 2.0 Fluorometer and the Qubit DNA HS assay (#Q32854) and the size distribution of fragments was assessed using the Agilent Bioanalyser with the DNA HS Kit (#5067-4626). Fragment size and quantity measurements were used to calculate molarity for each library pool.

Sequencing

Sequencing was performed using the NextSeq 500/550 High-Output v2 (150 cycle) Kit (# FC-404-2002) on the NextSeq 550 platform (Illumina Inc, #SY-415-1002)

2. Summary of Analysis Pipeline

Basecall data produced by the NextSeq 550 is automatically uploaded to BaseSpace, a cloud-based data management and analysis service provided by Illumina. Here it is converted into FASTQ files in order to allow analysis using a number of apps accessible directly through BaseSpace, or to download so that alternative analysis pipelines can be used.

3. Summary

A 2x75bp sequencing run on the Nextseq 550 using a high output flow cell is expected to generate up to 400M reads (50-60Gb) with a data quality of >80% higher than Q30, based on a cluster density of 170-230K/mm². When multiplexing 6 samples per flow cell we would therefore expect to see up to ~66M paired-end (PE) reads per sample. Table 1 below summarises the sequencing metrics for each run.

Run ID	Cluster Density (K/mm²)	% Clusters PF	Yield (Gb)	% Data > Q30
170428_NB551016_0077_AHCVFWBGX2	28/04/2017	191	93.7	71.9
170503_NB551016_0079_AHCMVNBGX2	03/05/2017	216	91.8	79.6
170505_NB551016_0080_AHCW3YBGX2	05/05/2017	216	93.1	80.9
170725_NB551016_0093_AHNTYWBGX2	25/07/2017	207	94.1	78.2
170726_NB551016_0094_AHNTT5BGX2	26/07/2017	235	92.7	87.7
170727_NB551016_0095_AH5VFFBGX3	27/07/2017	239	93.2	89.5
170728_NB551016_0096_AH5V7FBGX3	28/07/2017	241	93.5	90.5
170731_NB551016_0097_AH7K3CBGX3	31/07/2017	252	91.8	93.1

All flow cells performed above expectations, with each producing >70Gb data (Min: 71.9Gb, Max: 93.1Gb, Mean: 83,7Gb). Data quality was high with at least 91% ≥Q30 for all runs. Coverage of each library was variable (Min: 30.1M, Max: 197.4M, Mean: 89.6M), though the majority of libraries generated >60M reads. Figure 1 below shows the distribution of reads generated per library.

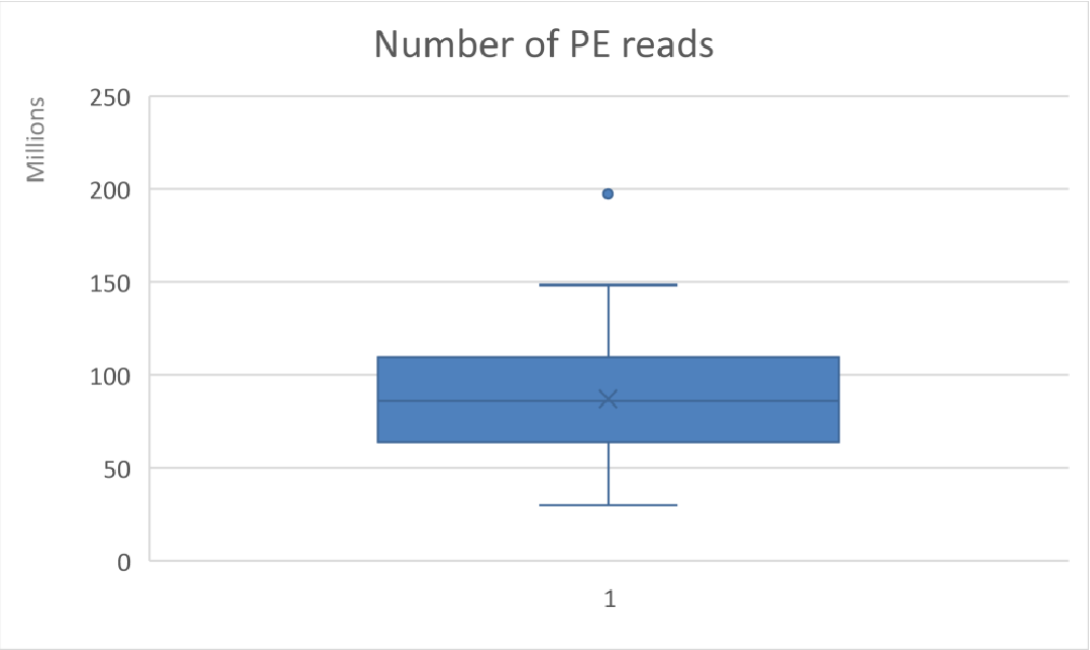

Figure 1: Number of paired-end (PE) generated per library. The box and whiskers show the 4 quartiles, and the line dividing the box shows the median. The x marks the mean. Points above or below the whiskers show outliers.

This variation in coverage is due in the main part to the nature of working with DNA extracted from FFPE tissue, where the quality of the nucleic acids recovered is dependent on many factors. These include:

- Intraoperative ischemia time
- Cold ischemia time
- Transport medium
- Time to fixation
- Type/time of fixation
- pH of formalin
- Specimen size (penetration of fixation)
- Type of processor
- Time in processor
- Hot wax temperature

DNA damage caused by the fixation process can include deamination of cytosine to uracil, oxidation, thymine dimers, nicks and double-strand breaks. Such damage impacts on the quality of sequencing libraries generated from FFPE DNA, and can make accurate quantification a challenge.

Table 2 below shows the number of PE reads generated per sample. Where a sample ID has an ‘R’ suffix this was a repeated library preparation.

Table 2: Library performance

Sample ID	Run ID	Sequence Date	Number of PE reads
4081	170428_NB551016_0077_AHCVFWBGX2	28/04/2017	39,625,791
4080	170428_NB551016_0077_AHCVFWBGX2	28/04/2017	109,940,900
4046	170428_NB551016_0077_AHCVFWBGX2	28/04/2017	61,155,592
320	170428_NB551016_0077_AHCVFWBGX2	28/04/2017	61,222,361
2210	170428_NB551016_0077_AHCVFWBGX2	28/04/2017	99,651,105
6595	170428_NB551016_0077_AHCVFWBGX2	28/04/2017	79,856,733
6611	170503_NB551016_0079_AHCMVNBGX2	03/05/2017	51,502,763
7012	170503_NB551016_0079_AHCMVNBGX2	03/05/2017	110,140,243
7523	170503_NB551016_0079_AHCMVNBGX2	03/05/2017	84,274,867
7707	170503_NB551016_0079_AHCMVNBGX2	03/05/2017	54,286,748
1341	170503_NB551016_0079_AHCMVNBGX2	03/05/2017	95,453,655
2070	170503_NB551016_0079_AHCMVNBGX2	03/05/2017	101,093,492
4486	170505_NB551016_0080_AHCW3YBGX2	05/05/2017	73,961,381
7136	170505_NB551016_0080_AHCW3YBGX2	05/05/2017	74,293,437
8315	170505_NB551016_0080_AHCW3YBGX2	05/05/2017	104,649,149
8343	170505_NB551016_0080_AHCW3YBGX2	05/05/2017	82,803,332
9241	170505_NB551016_0080_AHCW3YBGX2	05/05/2017	89,050,302
8082	170505_NB551016_0080_AHCW3YBGX2	05/05/2017	75,646,269
8500	170725_NB551016_0093_AHNTYWBGX2	25/07/2017	64,827,987
7920	170725_NB551016_0093_AHNTYWBGX2	25/07/2017	86,233,332
7060	170725_NB551016_0093_AHNTYWBGX2	25/07/2017	114,649,297
3874	170725_NB551016_0093_AHNTYWBGX2	25/07/2017	49,293,564
6662	170725_NB551016_0093_AHNTYWBGX2	25/07/2017	102,952,673
5573	170725_NB551016_0093_AHNTYWBGX2	25/07/2017	69,642,937
9542	170726_NB551016_0094_AHNTT5BGX2	26/07/2017	118,549,339
7379	170726_NB551016_0094_AHNTT5BGX2	26/07/2017	81,716,208
9310	170726_NB551016_0094_AHNTT5BGX2	26/07/2017	121,329,258
9295	170726_NB551016_0094_AHNTT5BGX2	26/07/2017	87,034,618
5433	170726_NB551016_0094_AHNTT5BGX2	26/07/2017	87,108,066
5864	170726_NB551016_0094_AHNTT5BGX2	26/07/2017	53,436,282
7865	170727_NB551016_0095_AH5VFFBGX3	27/07/2017	75,952,086
8058	170727_NB551016_0095_AH5VFFBGX3	27/07/2017	98,459,382
22110	170727_NB551016_0095_AH5VFFBGX3	27/07/2017	142,099,679
7902	170727_NB551016_0095_AH5VFFBGX3	27/07/2017	98,382,359
7972	170727_NB551016_0095_AH5VFFBGX3	27/07/2017	115,632,732
1695	170727_NB551016_0095_AH5VFFBGX3	27/07/2017	30,480,403
8086	170728_NB551016_0096_AH5V7FBGX3	28/07/2017	71,961,003
21899R	170728_NB551016_0096_AH5V7FBGX3	28/07/2017	63,613,102
9231	170728_NB551016_0096_AH5V7FBGX3	28/07/2017	85,540,452
4629	170728_NB551016_0096_AH5V7FBGX3	28/07/2017	46,962,091
21729	170728_NB551016_0096_AH5V7FBGX3	28/07/2017	197,457,027
4229	170728_NB551016_0096_AH5V7FBGX3	28/07/2017	100,917,163

22106	170731_NB551016_0097_AH7K3CBGX3	31/07/2017	113,285,259
21860	170731_NB551016_0097_AH7K3CBGX3	31/07/2017	83,205,087
9079	170731_NB551016_0097_AH7K3CBGX3	31/07/2017	111,900,966
9305	170731_NB551016_0097_AH7K3CBGX3	31/07/2017	85,818,784
21770	170731_NB551016_0097_AH7K3CBGX3	31/07/2017	143,556,178
3817	170731_NB551016_0097_AH7K3CBGX3	31/07/2017	45,925,418
9332	170809_NB551016_0104_AHGWCNBGX3	09/08/2017	132,901,071
3785	170809_NB551016_0104_AHGWCNBGX3	09/08/2017	91,906,569
8460	170809_NB551016_0104_AHGWCNBGX3	09/08/2017	77,421,286
9735	170809_NB551016_0104_AHGWCNBGX3	09/08/2017	91,013,173
587R	170809_NB551016_0104_AHGWCNBGX3	09/08/2017	55,745,643
9693	170809_NB551016_0104_AHGWCNBGX3	09/08/2017	116,017,105
9801	170810_NB551016_0105_AHGV7CBGX2	10/08/2017	113,548,429
22299	170810_NB551016_0105_AHGV7CBGX2	10/08/2017	148,534,366
9759	170810_NB551016_0105_AHGV7CBGX2	10/08/2017	82,434,839
4006	170810_NB551016_0105_AHGV7CBGX2	10/08/2017	76,983,600
22270	170810_NB551016_0105_AHGV7CBGX2	10/08/2017	106,138,899
5071	170810_NB551016_0105_AHGV7CBGX2	10/08/2017	36,103,387
8429	170811_NB551016_0106_AHGMG7BGX3	11/08/2017	64,033,496
8462	170811_NB551016_0106_AHGMG7BGX3	11/08/2017	46,688,885
21776	170811_NB551016_0106_AHGMG7BGX3	11/08/2017	112,135,557
3362	170811_NB551016_0106_AHGMG7BGX3	11/08/2017	109,294,761
4319	170811_NB551016_0106_AHGMG7BGX3	11/08/2017	38,816,160
21636	170811_NB551016_0106_AHGMG7BGX3	11/08/2017	120,837,074
5183	171201_NB551016_0136_AH23HVBGXJ	01/12/2017	141,916,015
21562	171201_NB551016_0136_AH23HVBGXJ	01/12/2017	94,853,975
9610	171201_NB551016_0136_AH23HVBGXJ	01/12/2017	105,651,098
1695R	171201_NB551016_0136_AH23HVBGXJ	01/12/2017	30,086,676
4080R	171201_NB551016_0136_AH23HVBGXJ	01/12/2017	39,423,393
5071R	171201_NB551016_0136_AH23HVBGXJ	01/12/2017	57,057,438

After the data from 11 runs were sequenced and analysed the Investigator selected three samples that had performed poorly for repeat library preparation and sequencing alongside the final 3 libraries (1695R, 4080R and 5071R). Performance of all three libraries was poor in relation to the other libraries in the same sequencing run (5183, 21562 and 9610) with all three repeats failing to generate >60M reads, suggesting that performance issues may be sample related.

7.2 Appendix B

List of commonly mutated genes previously reported in either endometrial, ovarian or pan-cancer studies.

No.	Gene	No.	Gene	No.	Gene
1	*AKT1*	36	*JAK3*	71	*TERT*
2	*AKT2*	37	*KDR*	72	*TP53*
3	*ALK*	38	*KIT*	73	*TSC1*
4	*APC*	39	*KRAS*	74	*USP9X*
5	*AR*	40	*KSR1*	75	*WT1*
6	*ARAF*	41	*MAP2K1*		
7	*ARID1A*	42	*MAP2K2*		
8	*ARID1B*	43	*MATK*		
9	*ATM*	44	*MET*		
10	*BRAF*	45	*MLH1*		
11	*BRCA1*	46	*MSH2*		
12	*BRCA2*	47	*MSH6*		
13	*CCND1*	48	*MTOR*		
14	*CCND2*	49	*NF2*		
15	*CCND3*	50	*NFE2L2*		
16	*CD274*	51	*NRAS*		
17	*CDK4*	52	*NTRK1*		
18	*CDK6*	53	*PAK1*		
19	*CDKN2A*	54	*PDCD1LG*		
20	*CTNNB1*	55	*PDGFRA*		
21	*EGFR*	56	*PDGFRB*		
22	*ERBB2*	57	*PIK3CA*		
23	*ESR1*	58	*PIK3R1*		
24	*EZH2*	59	*PMS2*		
25	*FBXW7*	60	*POLE*		
26	*FGFR1*	61	*PRKD1*		
27	*FGFR2*	62	*PTCH1*		
28	*FGFR3*	63	*PTEN*		
29	*FLT1*	64	*RAF1*		
30	*FLT3*	65	*RB1*		
31	*FLT4*	66	*RET*		
32	*GATA3*	67	*RNF43*		
33	*IDH1*	68	*ROS1*		
34	*IDH2*	69	*SMAD4*		
35	*JAK2*	70	*STK11*		

7.3 Appendix C

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Endocrine treatment of high grade serous ovarian carcinoma; quantification of efficacy and identification of response predictors

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HIGHLIGHTS

- Endocrine therapy has efficacy in relapsed high grade serous ovarian cancer.
- It can be used to delay subsequent chemotherapy.
- Those with ER H-score > 200 and treatment free interval > 180 days are most likely to benefit.

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ABSTRACT

Objectives. The role of endocrine therapy (ET) in high grade serous ovarian carcinoma (HGSOC) is poorly defined due to the lack of phase III data and significant heterogeneity of clinical trials performed. In this study, we sought to identify predictive factors of endocrine sensitivity in HGSOC.

Methods. HGSOC patients who received at least four weeks of ET for relapsed disease following one line of chemotherapy at the Edinburgh Cancer Centre were identified. Exclusion criteria were use of endocrine therapy as maintenance therapy or of unknown duration. Duration of therapy and best CA125 response as per modified GCIG criteria were recorded. Oestrogen receptor (ER) histo score, treatment free interval, prior lines of chemotherapy, and type of ET were evaluated as predictive factors.

Results. Of 431 patients identified, 269 were eligible (77.0% letrozole, 18.6% tamoxifen, 2.2% megesterol acetate, 2.2% other). The median duration of therapy was 126 days (range 28–1427 days). 32.7% remained on ET for ≥180 days and 14.1% for ≥365 days. The CA125 response and clinical benefit rates (response or stable disease) were 8.1% and 40.1% respectively. ER histoscore > 200 ($P = 0.0016$) and a treatment free interval of ≥180 days ($P < 0.0001$) were independent predictive factors upon multivariable analysis.

Conclusions. ET should be considered as a viable strategy to defer subsequent chemotherapy for relapsed HGSOC. Patients with an ER histoscore > 200 and a treatment free interval of ≥180 days are most likely to derive benefit.

1. Introduction

The majority of patients with advanced stage high grade serous ovarian carcinoma (HGSOC) will unfortunately relapse despite optimal cytoreductive surgery and platinum based chemotherapy. Symptomatic relapses are treated with further systemic chemotherapy which can be effective for some patients. However, with time, the intervals between each treatment get progressively shorter with reduced efficacy and cumulative toxicity.

Endocrine therapy (ET) in relapsed HGSOC is easy to administer, has a low toxicity profile and is low cost. There is good pre-clinical evidence to support the role of oestrogen in regulating the growth of oestrogen receptor (ER) positive EOC [1,2]. To date, >50 phase II trials of ET in EOC have been performed with response rates between 10 and 15% and disease stabilisation rates of 30–40% as described in systematic reviews and meta-analyses [3,4]. Only one phase III randomised trial of

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7.4 Appendix D

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Hormone receptor expression patterns define clinically meaningful subgroups of endometrioid ovarian carcinoma

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HIGHLIGHTS

- We identify four subgroups of endometrioid ovarian carcinoma (EnOC) defined by hormone receptor expression patterns.
- EnOC patients in the PR^{high} (PR+/ER+, PR+/ER−) groups demonstrate markedly favorable outcome.
- Stage II EnOC patients in the PR^{high} groups display a ten-year survival of approximately 95%.
- ER^{high} (PR+/ER+, PR−/ER+) EnOC patients had a higher body mass index vs ER^{low} cases and high grade serous carcinoma patients.

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ABSTRACT

Background: Numerous studies have investigated the association between hormone receptor expression and clinical outcome in ovarian carcinoma (OC); however, these have largely focussed on serous OCs, with few studies reporting specifically on endometrioid OCs (EnOC). Where analyses have been stratified by histotype, expression has been assessed using the percentage of positive tumor cells, without accounting for nuclear expression intensity.

Methods: Here we assess the expression levels of progesterone receptor (PR), estrogen receptor alpha (ER) and androgen receptor (AR) using histoscore – a nuclear scoring method incorporating both proportion of positive cells and the intensity of nuclear staining – across a cohort of 107 WT1 negative EnOCs.

Results: Hierarchical clustering by PR, ER and AR histoscores identified four EnOC subgroups (PR+/ER+, PR+/ER−, PR−/ER+ and PR−/ER−). EnOC patients in the PR+/ER+ and PR+/ER− groups displayed favorable outcome (multivariable HR for disease-specific survival 0.05 [0.01–0.35] and 0.05 [0.00–0.51]) compared to the PR−/ER+ group. Ten-year survival for stage II PR^{high} and PR^{low} cases was 94.1% and 42.4%. ER^{high} EnOC patients (PR+/ER+, PR−/ER+) had higher body mass index compared to ER^{low} cases ($P = 0.015$) and high grade serous OC patients ($P < 0.001$).

Conclusion: These data demonstrate that endometrioid OC cases with high PR expression display markedly favorable outcome. Stage II EnOCs with high PR expression represent potential candidates for de-escalation of first-line therapy. Future work should seek to characterise the sensitivity of PR and ER positive EnOCs to endocrine therapy.

1. Introduction

Ovarian cancer is the most lethal cancer of the female genital tract, and accounts for over 180,000 deaths per year worldwide [1]. The vast majority of cases are ovarian carcinomas (OCs), comprising five core histological subtypes: high grade serous (HGS), endometrioid, clear cell, low grade serous (LGS) and mucinous OC [2].

Endometrioid OCs (EnOCs) account for approximately 10% of cases, are associated with favorable prognosis compared to other histotypes, and are often diagnosed at FIGO stage I or II [2–4]. EnOCs are frequently estrogen receptor alpha (ER) and/or progesterone receptor (PR)-positive, and display WT1 negativity [5]. The routine use of WT1 immunohistochemistry (IHC) is known to

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